Reaction of variant sperm-whale myoglobins with hydrogen peroxide: the effects of mutating a histidine residue in the haem distal pocket

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The reaction of hydrogen peroxide with a number of variants of sperm-whale myoglobin in which the distal pocket histidine residue (His^{64}) had been mutated was studied with a combination of stopped-flow spectroscopy and freeze-quench EPR. The rate of the initial bimolecular reaction with hydrogen peroxide in all the proteins studied was found to depend on the polarity of the amino acid side chain at position 64. In wild-type myoglobin there were no significant optical changes subsequent to this reaction, suggesting the rapid formation of the well-characterized oxyferryl species. This conclusion was supported by freeze-quench EPR data, which were consistent with the pattern of reactivity previously reported [King and Winfield (1963) J. Biol. Chem. **238**, 1520–1528]. In those myoglobins bearing a mutation at position 64, the initial bimolecular reaction with hydrogen peroxide yielded an intermediate species that subsequently decayed via a second hydrogen peroxide-dependent step leading to modification or destruction of the haem. In the mutant $His^{64} \rightarrow Gln$ the calculated electronic absorption spectrum of the intermediate was not that of an oxyferryl species but seemed to be that of a low-spin ferric haem. Freeze-quench EPR studies of this mutant and the apolar mutant ($His^{64} \rightarrow Val$) revealed the accumulation of a novel intermediate after the first hydrogen peroxide-dependent reaction. The unusual EPR characteristics of this species are provisionally assigned to a low-spin ferric haem with bound peroxide as the distal ligand. These results are interpreted in terms of a reaction scheme in which the polarity of the distal pocket governs the rate of binding of hydrogen peroxide to the haem iron and the residue at position 64 governs both the rate of heterolytic oxygen scission and the stability of the oxyferryl product.

INTRODUCTION

The reaction of myoglobin with hydrogen peroxide has been a subject of study over many years from such perspectives as: (1) a possible source of oxidative damage in tissue subsequent to such events as myocardial reperfusion after ischaemia [1–3], (2) a model for some of the reactions catalysed by peroxidases and catalase [4], and (3) a catalyst for the oxidation chemistry of a number of small organic molecules such as styrene [5]. These studies have identified the formation of a ferryl species [6–8], a radical species [9–11] and specific roles for a number of amino acid residues within the protein [5,12–14].

We have had a particular interest in the possible role of the amino acid histidine that occurs at position 64 in the amino acid sequence of sperm-whale myoglobin; it blocks the access channel of small molecules into the haem crevice [15–18] and is close to the iron atom of the haem on the distal side. This residue is largely responsible for the control of the co-ordination chemistry of the haem group. In metmyoglobin (the oxidized form of the protein) His^{64} hydrogen-bonds to a water molecule bound to the haem iron; in the oxygenated form of the protein it contributes significantly to the stabilization of the haem-bound dioxygen [19–21]. Furthermore this amino acid has been implicated in the control of the chemical reactions that can occur between peroxide-treated myoglobin and small unsaturated organic molecules [22]. It has been considered to take part directly in epoxidation reactions and also to act as a mediator of electron transfer of unpaired electron density, which is responsible for free radical transfer to other amino acid sites within the protein [5].

In this paper we report on the reaction of a number of site-directed mutant forms of the protein, altered at position 64, with hydrogen peroxide. We have investigated the kinetics of these reactions with stopped-flow spectrophotometry and EPR spectroscopy. The data have been rationalized in terms of a plausible mechanism in which the chemical nature of the residue at position 64 within the protein sequence has a pivotal role in the control of the relative rates of the various processes in operation and the stability of the oxyferryl intermediate.

MATERIALS AND METHODS

Cell growth, protein purification and characterization

The *Escherichia coli* strain TB1 was transformed with a plasmid containing a synthetic gene that encodes either the wild-type sperm-whale myoglobin or a variant carrying a mutation at His^{64}. Conditions for batch cell culture and subsequent purification of the expressed myoglobins were as previously described [23].

To ensure that the purified variant myoglobins carried the expected mutation, samples of each were analysed by electrospray ionization MS. Mass spectra were taken on a Fisons VG Platform with CH_3CN/water/formic acid (1:1:0.0001, by vol.) as sample solvent and horse heart myoglobin as a standard. Samples were dialysed against 0.1% (v/v) formic acid or 1 mM Tris/HCl, pH 7.5, to remove salts and mixed with the sample solvent immediately before analysis. Approximately 30 pmol of protein was used per run. Data were acquired over the range m/z 700–1300; five or more scans were averaged and processed with the supplier's MassLynx software. The typical S.D. for each mass determination was 1.0–2.0 Da and the observed mass of the proteins was typically within 1.5 Da of the calculated value.

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Sample preparation

To ensure that the proteins used in the experiments were all in the ferric form, each sample was oxidized by the addition of a small excess of potassium ferricyanide. Excess oxidant was subsequently removed by gel filtration on a small column (5 cm × 1.6 cm) packed with Sephadex G-25 equilibrated with the reaction buffer.

Spectroscopy

EPR spectra were recorded on an X-band ER 200D spectrometer (Bruker Spectrospin) interfaced to an ESP 1600 computer and fitted with a liquid helium flow-cryostat (ESR-9; Oxford Instruments). Samples of $His^{64} \rightarrow Val$ were mixed with excess hydrogen peroxide in an EPR cuvette and the reaction was rapidly quenched by immersing the cuvette in liquid nitrogen. Samples of wild-type myoglobin and $His^{64} \rightarrow Gln$ were mixed with excess hydrogen peroxide on a millisecond time scale by using the freeze-quench apparatus previously described [24].

Kinetic measurements

The reaction of the various metmyoglobins with hydrogen peroxide under pseudo-first-order conditions was measured in an Applied Photophysics Bio-Sequential DX.17MV stopped-flow spectrophotometer with a 1 cm path-length cell. Detection at a single wavelength was with a side-window photomultiplier. In this configuration a minimum of 500 data points were collected per experiment. Detection at multiple wavelengths was achieved with an Applied Photophysics photodiode array accessory. A minimum of 200 spectra were collected per experiment with a maximum time resolution of 2.38 ms per spectrum.

Treatment of kinetic data

The experimental traces recorded at 430 nm were exported as ASCII files and analysed as the sum of two or three exponentials with TableCurve 2D for Windows (Jandel Scientific, San Rafael, CA, U.S.A.). A variety of kinetic mechanisms were investigated to determine whether they could account for the observed kinetic characteristics exhibited by the reaction of metmyoglobin with hydrogen peroxide. Each mechanism was explored in the time domain by using the Gear variable-step numerical integration method to generate theoretical time courses [25]. The time courses were then fitted to the experimental data by varying the rate constants in the scheme being tested. When an adequate mechanism had been found, the theoretical time courses were synthesized for hydrogen peroxide concentrations from 0.1 to 90 mM to allow comparison with the experimentally determined concentration dependence.

The time-resolved spectra were analysed globally at all times and all wavelengths simultaneously. The analysis was performed with an Acorn A5000 personal computer by using singular value decomposition and global exponential-fitting routines found in the software package Glint (Applied Photophysics, Leatherhead, Surrey, U.K.).

RESULTS AND DISCUSSION

Changes in the electronic absorption spectrum on reaction with hydrogen peroxide

Both wild-type sperm-whale myoglobin and each of the His^{64} mutants were mixed rapidly with 90 mM hydrogen peroxide in the stopped-flow apparatus and the progress of the reaction was followed with a photodiode array detector. In the wild-type protein the reaction is essentially described by a single-exponential process (results not shown) at all wavelengths monitored. The spectral changes observed in response to the reaction with hydrogen peroxide are similar to those previously reported [10]. There is a slight red-shift and increase in intensity in the Soret region, whereas in the visible region the 640 nm charge transfer band, which is a marker of high-spin ferric haem, disappears and there is an increase in absorbance at 550 and 585 nm. This new species has previously been shown by NMR [6], resonance Raman [7] and magnetic CD [26] spectroscopies to contain oxyferryl haem.

When hydrogen peroxide reacts with the polar mutant $His^{64} \rightarrow Gln$ (Figure 1A) there is a progressive loss of absorbance over the entire Soret region and on longer time scales a significant increase in absorbance centred on 700 nm in the visible region of the spectrum. The reaction time course seems to consist of two exponential processes that are most easily visualized at 430 nm (Figure 2a). All the mutant myoglobins except $His^{64} \rightarrow Tyr$ showed a similar qualitative pattern of reactivity towards hydrogen peroxide, although the time scale of the reactions varied by nearly three orders of magnitude (Figure 2 and Table 1).

Effect of hydrogen peroxide concentration on the time course of the reaction

The dependence on hydrogen peroxide concentration of the observed rate constants associated with each of the kinetic phases described above was examined. Both phases of the reaction seemed to show a linear dependence on the concentration of hydrogen peroxide. A plot of k_{obs} of the fastest phase as a function of hydrogen peroxide concentration not only demonstrates this relationship (Figure 3) but also reveals that the line of best fit has a non-zero intercept with the y-axis in each of the proteins examined. Because under pseudo-first-order conditions the y-intercept provides an estimate of k_{off}, it was clear that each of these processes showed a significant back reaction. This also proved true of the single-exponential process observed for the reaction of hydrogen peroxide with native and recombinant wild-type metmyoglobin (Table 1).

The derived equilibrium constants (Table 1) are suggestive of reversible peroxide ion binding to the ferric iron centre. Although peroxide binding at the iron is weak, the relative rates of reaction of hydrogen peroxide with the protein vary by nearly three orders of magnitude (Table 1) and seem to depend on the polarity of the residue at position 64. This suggests that hydrogen peroxide needs to be deprotonated before binding to the haem and that this process is aided by the presence of a polar residue at position 64. A similar argument has been advanced for the binding of cyanide to metmyoglobins [27,28]. However, the relative rates of reactivity with cyanide for a series of myoglobins bearing mutations at position 64 are not the same as those with hydrogen peroxide and might reflect different mechanisms of ligand deprotonation.

Calculation of the electronic absorption spectrum of the reaction intermediate

The rate constants presented in Table 1 allowed us to identify experimental conditions optimal for the resolution of the spectrum of the reaction intermediate and the final product. These conditions [3.62 μM metmyoglobin ($His^{64} \rightarrow Gln$), 90 mM hydrogen peroxide] were used in an experiment to measure the changes in the electronic absorption spectra (350–800 nm) associated with the sequential formation of the intermediate and the product.

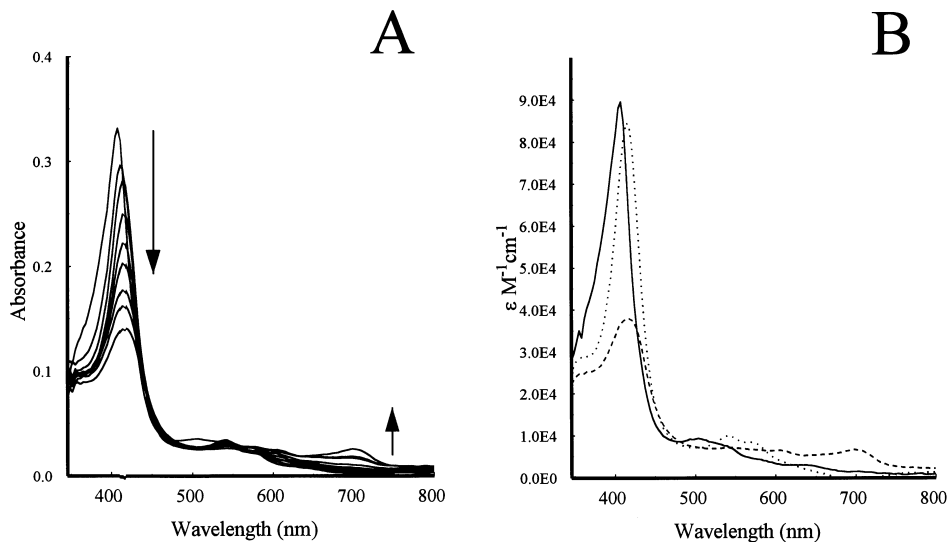


Figure 1 Spectral changes induced in myoglobin (His^{64} → Gln) on reaction with hydrogen peroxide

Spectral changes in the myoglobin mutant His^{64} → Gln after mixing with 90 mM hydrogen peroxide in 50 mM Tris/HCl buffer at pH 7.5 and 20 °C were monitored with a photodiode array detector with an integration time of 2.38 ms. A total of 200 spectra were recorded over 0.5 s. (**A**) Representative spectra recorded at the following times; 1.3, 14, 27, 52, 78, 104, 155, 206 and 437 ms. Arrows indicate the direction of absorbance changes with time. (**B**) Spectra of the authentic starting material and the reaction intermediates calculated by a combination of singular value decomposition and global analysis as described in the text.

After reduction of the data set by singular value decomposition [29] to the eight most significant components, the data were reconstructed and subjected to a global fitting routine [30]. The reaction was fitted to the following model:

$$\text{metmyoglobin} + H_2O_2 \rightarrow I \rightarrow II$$

Figure 2(a) shows the formation and decay of the intermediate monitored at 430 nm. This demonstrates that, as predicted, the occupancy of the intermediate state is maximal after 20 ms. The spectral changes associated with the formation and decay of the intermediate species are shown in Figure 1(A). These demonstrate several features: first, a progressive loss of intensity in the Soret region throughout the reaction; secondly, towards the end of the reaction a new feature appears at approx. 700 nm; thirdly, in the early part of the reaction some additional intensity around 540 and 560 nm is apparent that subsequently decays as the 700 nm band forms.

From this data set a global fitting routine was used to calculate the spectrum of the reaction intermediate and that of the product (Figure 1B). The spectrum of the starting material was constrained to that of authentic His^{64} → Gln metmyoglobin. Compared with the starting spectrum the calculated spectrum of the intermediate species exhibited a red-shifted Soret, a loss of intensity at 624 nm associated with a ligand to metal charge-transfer band that is a marker of high-spin ferric haem, and additional intensities at 540 and 562 nm. The spectrum of this intermediate is unlike that of the well-characterized product of the reaction of wild-type sperm-whale myoglobin with hydrogen peroxide, which is an oxyferryl species. Instead it seems to have more in common with the spectra of myoglobins containing low-spin ferric haem such as the cyanide and imidazole adducts [31].

The calculated spectrum of the product is different again. The red-shifted Soret region exhibits approx. 25 % the intensity of the starting material, with a broad maximum at 420 nm. The visible region is rather featureless and there is a new band in the near IR region centred at 700 nm. This spectrum is reminiscent of that of the soluble domain of rat liver haem oxygenase after treatment with a single equivalent of hydrogen peroxide, which yields enzyme-bound ferric verdohaem [32].

Changes in the EPR spectrum during the time course of the reaction

Having identified the existence of two optically distinct phases in the reaction of hydrogen peroxide with the mutant myoglobins by using rapid absorption spectroscopy, we wished to investigate the possibility that the intermediate we observed in the reaction was not an oxyferryl species but a low-spin ferric haem. To do this we chose examples bearing a polar substitution (His^{64} → Gln) and an apolar substitution (His^{64} → Val) for further investigation by EPR spectroscopy. On the basis of the rate constants given in Table 1 we chose to use 90 mM hydrogen peroxide because simulations predicted that the oxyferryl state would be maximally populated under these conditions, albeit in very different time domains (Figure 2).

As a control we measured the EPR spectra associated with the reaction mixtures of recombinant wild-type myoglobin and hydrogen peroxide. This revealed the participation of only two EPR-active species (Figure 4B). The initial $g = 6$; $g = 1.98$ species diagnostic of high-spin ferric haem disappears and is replaced by a radical species. This we presume to be that initially reported by King et al. [11] and later assigned to a Tyr^{151} radical [5,12,13]. This radical Tyr^{151} has been suggested to be a secondary radical site originating from initial proton abstraction from His^{64} in the wild-type protein [5]. The radical species then slowly disappears and is replaced by an EPR-silent form of the protein, which would be consistent with formation of oxyferryl haem and quenching of the radical at the surface of the protein.

In contrast, in both mutants studied the signals associated with high-spin ferric haem are replaced by a new rhombic trio of

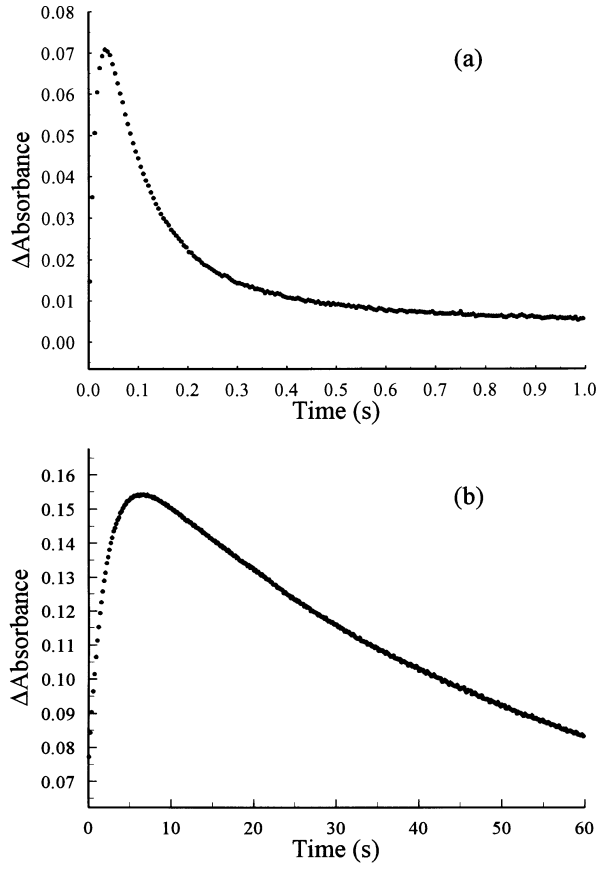


Figure 2 Time courses for the reaction of myoglobin with hydrogen peroxide

The time course is shown for the reaction of myoglobin mutant $His^{64} \rightarrow Gln$ (**a**) and $His^{64} \rightarrow Val$ (**b**) with 90 mM hydrogen peroxide in 50 mM Tris/HCl buffer at pH 7.5 and 20 °C, followed at 430 nm.

signals at $g = 2.29$; $g = 2.16$ and $g = 1.91$ (Figures 4A and 4C) that we ascribe to low-spin ferric haem with histidine and peroxide axial ligation (see below). In the polar mutant $His^{64} \rightarrow Gln$ a radical species similar to that seen in the wild-type protein forms on approximately the same time scale. Subsequently this dissipates and a new signal attributable to ferric non-haem iron appears at $g = 4.3$, which is evidence of haem destruction. A

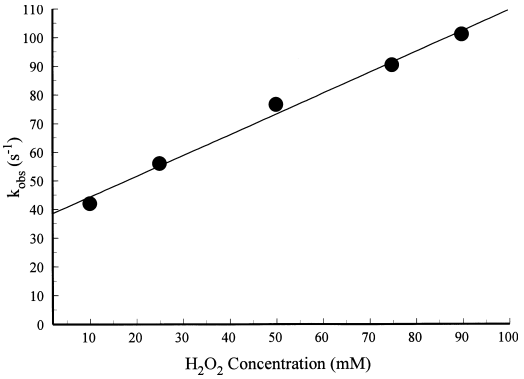


Figure 3 Concentration dependence of the fast kinetic processes observed in the reaction of myoglobin with hydrogen peroxide

The concentration dependence of the fastest kinetic phase observed in the reaction of myoglobin mutant $His^{64} \rightarrow Gln$ with hydrogen peroxide is shown. The reaction conditions were as for Figure 1 (see also Table 1).

qualitatively similar pattern of reactivity is shown by the apolar mutant $His^{64} \rightarrow Val$ on a much longer time scale.

The nature of the 'peroxy' intermediate

The rhombic EPR spectrum ($g = 2.29$; $g = 2.16$; $g = 1.91$) found in the reaction mixtures quenched during the early stages of the reactions between both the $His^{64} \rightarrow Gln$ and $His^{64} \rightarrow Val$ mutants and hydrogen peroxide has not previously been reported (Figure 5). Because the appearance of this EPR-active species corresponds to the product of the initial bimolecular reaction of hydrogen peroxide with the variant myoglobins we presume that it corresponds to a peroxy intermediate. An oxyferryl species would either be EPR-silent or, if it contained a π-cation radical on the porphyrin macrocycle, it might manifest itself as a symmetric radical.

Sperm-whale myoglobin containing ferric haem with histidine and hydroxide ion as axial ligands is low-spin at 10 K and gives rise to a rhombic spectrum, $g = 2.57$; $g = 2.14$; $g = 1.84$ [33]. We see no evidence for such a hydroxy species, nor for the rhombic species found in the reaction of horse heart myoglobin with hydrogen peroxide ($g = 2.035$; $g = 2.008$; $g = 2.001$). Taking this with the optical data presented above, we believe that the

Table 1 Kinetic parameters of wild-type and mutant myoglobins in their reaction with H_2O_2

The results for auto-oxidation rate are taken from [15].

Residue at position 64	Soret maximum (nm)	Bound water	Auto-oxidation rate (h^{-1})	k_1 ($M^{-1} \cdot s^{-1}$)	k_{-1} ($M^{-1} \cdot s^{-1}$)	K_1 (mM)	k_4 ($M^{-1} \cdot s^{-1}$)
Glutamine	408	Yes	0.21	728	36	50	140
Histidine (native)	409	Yes	0.06	234	1.8	8	–
Histidine (recombinant)	409	Yes	0.06	227	1.5	7	–
Glycine	408	Yes	44	47	0.3	6	3.4
Alanine	407	Yes	58	31	0.07	2	3.0
Threonine	397	?	–	8.4	0.02	2	0.08
Valine	395	No	33	3	0.05	17	0.11
Phenylalanine	395	No	6	1.5	0.05	33	< 0.05
Tyrosine	410	Phenol	> 100	–	–	–	–

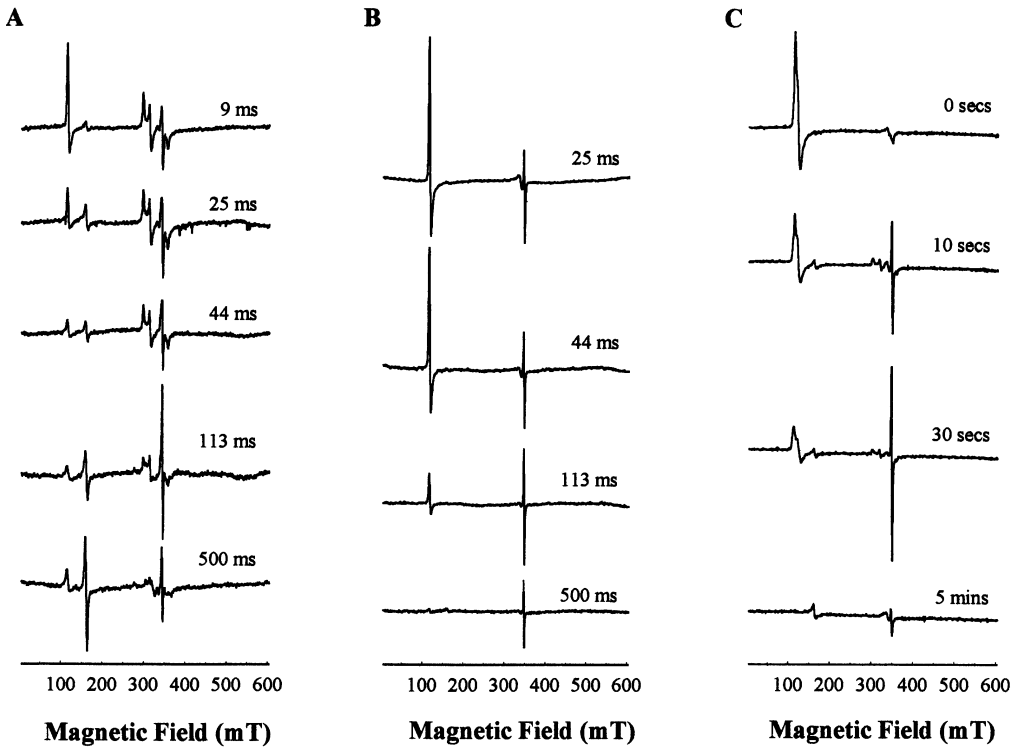


Figure 4 Time course of the development of X-band EPR signals associated with the reaction of myoglobin with hydrogen peroxide

The development of EPR signals after reaction of three different metmyoglobins with 90 mM hydrogen peroxide in 50 mM Hepes, pH 7.5: (**a**) $His^{64} \rightarrow Gln$; (**b**) wild-type; (**c**) $His^{64} \rightarrow Val$. The spectra shown are the average of three scans and the conditions of measurement were: (**A**) microwave frequency 9.59 GHz, microwave power 2.03 mW, temperature 10 K, modulation amplitude 804 μT; (**B**, **C**) microwave frequency 9.67 GHz, microwave power 2.03 mW, temperature 10 K, modulation amplitude 892 μT.

most reasonable assignment for these novel signals is that of low-spin ferric haem with a peroxide ion as the distal ligand.

The observation of an EPR signature associated with a haem-bound peroxide is important. Such a species has been proposed to participate in the reaction mechanism of peroxidases [34,35], haem–copper oxidases [36] and haem oxygenases [32], although the electronic absorption and EPR spectra associated with such a species have proved elusive. Recently, Rodriguez-Lopez et al. [34] have reported a spectrum with a blue-shifted Soret maximum in a mutant form of horseradish peroxidase in which the distal pocket Arg^{38} had been changed to a leucine residue. The assignment of this species as a bound neutral peroxide is supported by electronic structure and spectral calculations [37]. However, its appearance is so transient as to preclude trapping for examination by EPR spectroscopy.

The participation of a stable bound peroxide species in the catalytic cycle of the haem–copper oxidases is not resolved. Two intermediates known as P (peroxy) and F (oxyferryl) one oxidation level apart and with characteristic electronic absorption spectra [36] have been shown to be intermediates in a single turnover of cytochrome aa_3 [38]. Related species have been observed in the aa_3-600 type quinol oxidase from *Bacillus subtilis* [39] and in the cytochrome bo_3 quinol oxidase from *Escherichia coli* [40–42]. Of these species only the F forms of cytochrome aa_3 and cytochrome bo_3 have been unambiguously assigned to an oxyferryl species on the basis of resonance Raman [43] and magnetic CD spectroscopy [40] respectively.

The chemical nature of the P form remains unresolved and has recently been discussed in some detail by Ferguson-Miller and Babcock [44]. However, if P is a low-spin ferric-haem-bound peroxide species of the type described here, it should be visible by EPR only when the nearby Cu_B centre is reduced. Such circumstances have recently been identified in the reaction of fully reduced cytochrome aa_3 with dioxygen at low temperatures [38], where the intermediate designated P_R is thought to contain haem a_3 in the P state whereas Cu_B remains reduced.

A possible mechanism for the reaction of hydrogen peroxide with metmyoglobins

To reconcile the data that we have obtained from stopped-flow spectroscopy and freeze-quench EPR for both wild-type and mutant myoglobins, it is helpful to consider a possible reaction mechanism (Scheme 1). In the case of the wild-type myoglobin it seems that our data are entirely consistent with previous studies [10]. That is to say, the immediate products of the reaction with hydrogen peroxide are an oxyferryl haem and a radical species that subsequently dissipates. The rate of formation of the oxyferryl species is governed by the bimolecular rate constant k_1 and the first-order rate constant k_2. Under our conditions we make two assumptions: that the presence of an adjacent radical does not affect the spectrum of the oxyferryl species and that k_2 is at least two orders of magnitude greater than the observed rate constant for the peroxide-binding reaction. Under these conditions the peroxy state would never be populated and the only process that would be observed optically is the transition from metmyoglobin to the oxyferryl species. The migration of the radical to the surface of the protein and its subsequent quenching is easily observed by freeze-quench EPR spectroscopy (Figure 4B).

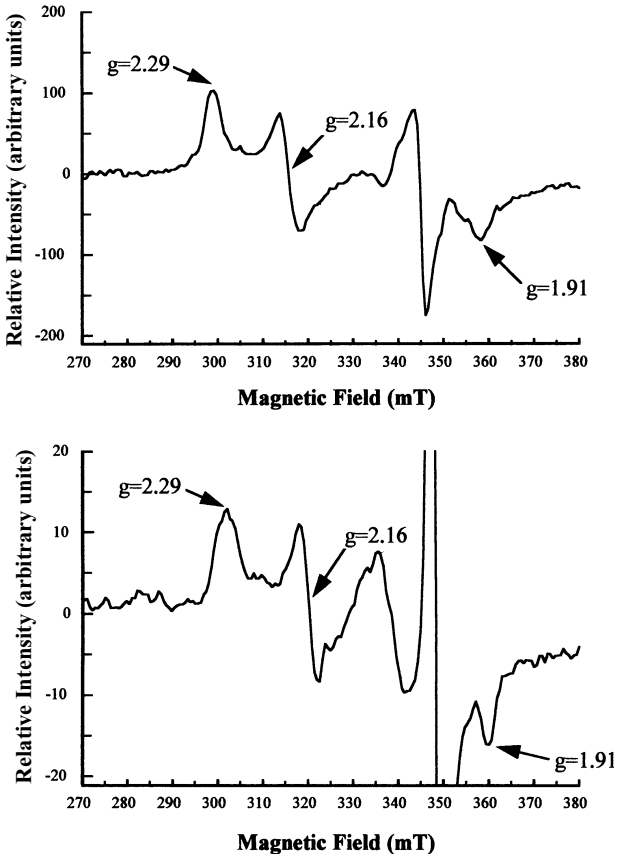
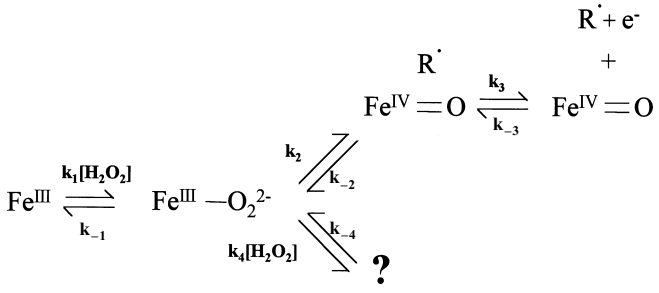


Figure 5 Detailed X-band EPR spectra of the initial product of the reaction of myoglobin with hydrogen peroxide

High-resolution spectra of the species present 25 ms ($His^{64} \rightarrow Gln$, upper panel) and 10 s ($His^{64} \rightarrow Val$, lower panel) after initiation of the reaction of hydrogen peroxide with myoglobin is shown. The conditions were as for Figure 4.



Scheme 1 Proposed mechanism for the reaction of wild-type and mutant myoglobins with H_2O_2

In the mutant myoglobins this is clearly not so. The absence of a histidine residue at position 64 seems to cause k_2 to be diminished to the extent that the rate of heterolytic oxygen cleavage becomes very sluggish. Under these circumstances the population of the 'peroxy' species would be controlled by k_1, k_{-1} and k_4, which represents the second peroxide-dependent process. Both the optical and the EPR data presented here provide evidence of the accumulation of a bound peroxide species in the mutants. In principle this reactive peroxide species could react with the porphyrin macrocyle and a second equivalent of peroxide to yield the species characterized by a bleached Soret band and the 700 nm band. Further evidence of haem destruction is found in the EPR spectra recorded at the end of the reaction time courses where there is evidence of non-haem iron. It is interesting to note that a bound peroxide species has been suggested as a catalytic intermediate in the formation of *α-meso*-hydroxyhaem by rat liver haem oxygenase, which is the immediate precursor of verdohaem [32].

The role of His^{64}

Once we have identified the basic pathways for the reaction of hydrogen peroxide with wild-type metmyoglobin and the mutants, it is important to consider the specific role of the amino acid side chain present at position 64 of the protein sequence. In the wild type a number of studies have identified two major roles for the distal histidine residue normally present at position 64. It can act as a swinging gate that controls access of the ligands to the distal pocket of the haem group [15]. Once the ligand has reached the distal pocket and bound to the haem iron, the N^{ϵ} of the imidazole ring can hydrogen-bond the polarized ligand and stabilize the iron–ligand complex [19]. In oxyferrousmyoglobin the Fe^{II} dioxygen bond is stabilized in this manner, as is the water molecule bound to the ferric haem in metmyoglobin [19]. It therefore seems likely that the imidazole ring is also responsible for stabilizing the oxyferryl species in wild-type myoglobin by acting as a hydrogen bond donor. If this is a role for His^{64} then perhaps the pH dependence of the electronic absorption and magnetic CD spectra of myoglobin is modulated by the protonation state of this residue rather than by the protonation state of the proximal histidine ligand as has been suggested previously [26].

A second consequence of changing His^{64} is to moderate the rate at which metmyoglobins can react with hydrogen peroxide. In the $His^{64} \rightarrow Tyr$ mutant the distal tyrosine residue has been shown to act as a 'permanent' ligand [19,45] and shows no reactivity towards peroxide (Table 1). However, the wild-type protein and the remaining six mutants show widely differing reactivities towards hydrogen peroxide. The pattern of reactivity does not correlate with the autoxidation rate (Table 1) but seems to correlate strongly with the polarity of the distal pocket and the level of water co-ordination to the haem iron in the ferric state.

The final effect of changing His^{64} is to impair heterolytic oxygen cleavage, allowing the accumulation of a reactive peroxide species. Because another polar residue (glutamine) seems unable to fulfil the same role, the implication is that heterolytic oxygen cleavage requires not only a proximal base but one that is precisely orientated with respect to the bound peroxide. To some extent this reflects the facile nature of this reaction in globins because in the authentic peroxidases yeast cytochrome *c* peroxidase and horseradish peroxidase the mutation of the distal histidine leads to an impairment in the rate of heterolytic oxygen cleavage but no change in the stability of the oxyferryl product compound I [35,46,47]. In these cases it is likely that a highly conserved proximal arginine residue contributes not only to rapid heterolytic oxygen cleavage but also to the stability of compound I. Globins lack a residue equivalent to Arg^{48} of yeast cytochrome *c* peroxidase; however, when this residue or the equivalent residue (Arg^{38}) in horseradish peroxidase is changed, heterolytic oxygen cleavage still takes place three orders of magnitude faster than in metmyoglobin [35,46,48]. This is probably due to the substantially different character of the proximal histidine residue in the peroxidases [49]. Nevertheless it seems clear that one role of a distal histidine residue in globins, peroxidases and perhaps oxidases is to prevent the accumulation

of reactive bound peroxide species that might otherwise cause oxidative damage to the prosthetic group.

Conclusions

Our experiments indicate some important considerations governing the reactivity of high-spin ferric haem proteins with hydrogen peroxide. First of all it seems that the dissociation constant for peroxide binding to the iron is rather large, which is perhaps indicative of a weakly bound species. The second point is that the rate at which peroxide binds to the haem is largely dependent on the polarity of the distal pocket and perhaps suggests that the reactive species is peroxide ion. Finally we show that in the absence of the distal histidine the immediate product of the reaction of metmyoglobin with hydrogen peroxide is not a stable oxyferryl haem but a reactive bound peroxide species that can cause oxidative damage to the porphrin macrocycle.

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