The Kinetics of the Reaction of Aquo Fe(III) Myoglobin with Hydrogen Peroxide at pH 8

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The kinetics of the reaction of aquo Fe(III) myoglobin (Mb(III)) with H₂O₂ at pH 8.0 and 20 °C have been investigated quantitatively under non-linear second order conditions. The data obtained at five concentrations of H₂O₂ (molar ratio [H₂O₂]/[Mb] total in the range 0.3 to 4) were analysed simultaneously by means of non-linear numerical integration and optimization techniques. The only mechanism that satisfied the triple requirement of a standard deviation within the standard error of the experimental data, good determination of the optimized parameters and a random distribution of residuals is as follows: Mb(III) reacts with H₂O₂ to form species X with a second order rate constant \( k_{+1} = 3.53 \times 10^2 \text{ M}^{-1} \text{ s}^{-1} \); species X in turn reacts with H₂O₂ to form species R with a second order rate constant \( k_{+2} = 3.40 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \); species R is then converted back into Mb(III) with a first order rate constant \( k_{+3} = 9.60 \times 10^{-1} \text{ s}^{-1} \). At 408 nm \( \Delta e_{\text{Mb(III)-R}} = 71.9 \text{ mM}^{-1} \text{ cm}^{-1} \) and \( \Delta e_{\text{Mb(III)-X}} = 104 \text{ mM}^{-1} \text{ cm}^{-1} \). Species R is spectrally identical to the Fe(IV) myoglobin species described by George and Irvine [Biochem. J., 52, 511–517 (1952)]. The chemical nature of species X and R is discussed on the basis of the data in the present paper and data in the literature from EPR, Mössbauer, resonance Raman and magnetic susceptibility studies.

Introduction

As part of our continuing study on the reactivity of haem groups in simple redox reactions and the manner in which their reactivity may be modified by combination with different apoproteins [1–3], we have examined in detail the kinetics of the reaction of aquo Fe(III) myoglobin (Mb(III)) with H₂O₂.

The reactions of a number of haem proteins with H₂O₂ have been described and are well characterized in the case of horseradish peroxidase [4], cytochrome c peroxidase [5] and catalase [6], but only poorly so in the case of Mb(III). Spectroscopic [7, 8] and magnetic susceptibility [9] studies indicate that Fe(IV) species are formed, and kinetic investigations have shown that the apparent second order rate constants for the formation of these species differ by up to a factor of 10⁵ within this series of haem proteins [10].

The reaction of Mb(III) with H₂O₂ in the pH range 8 to 9 results in the formation of a relatively stable red compound which we will call species R and which is characterized by an absorption maximum at 422 nm in the Soret region, together with a maximum at 547 nm, a weak band at 580–590 nm and a low shoulder at 510–520 nm in the a, β region [11–13]. At pH values below 8, a green compound is also formed and the products of the reaction are unstable; above pH 9, species R is converted to choleglobin in which the porphyrin ring has been oxidized [11, 12].

Redox titrations on species R demonstrated that the iron atom retained one oxidizing equivalent above the ferric state [11, 14]. Magnetic susceptibility [9], resonance Raman [8] and Mössbauer [7] studies on species R are consistent with the formulation of an Fe(IV) low spin, axially compressed t₂g system in a ³A₂ ground state. The ligand in the sixth coordination position of the iron atom of species R is unknown.

The kinetics of the reaction of Mb(III) with H₂O₂ between pH 8 and 9 have been studied by a number of other workers, and although not fully characterized and understood, shown to be complex [11, 14–16]. However, a number of features of the reaction are known which put certain constraints on any proposed kinetic scheme. First, the complete conversion of Mb(III) to species R requires more than an equimolar amount of H₂O₂, and the relative amount of species R formed is dependent on the molar ratio of H₂O₂ to myoglobin and independent of the absolute concentrations of the reactants [11]. On the basis of these data George and Irvine [11] concluded that the formation of species R from Mb(III) and H₂O₂ proceeds by an irreversible process and does not occur by a single step mechanism. Second, under conditions of approximately stoichiometric concen-
trations of reactants, the formation of a transient free radical species has been detected by EPR [14-16].

Third, O$_2$ is evolved and Mb(III) regenerated during the course of the reaction [15].

In the present paper we have analysed the kinetics of the reaction of Mb(III) with H$_2$O$_2$ at pH 8.0 under non-linear second order conditions (molar ratio [H$_2$O$_2$]/[Mb]$_{total}$ in the range 0.3 to 4) quantitatively by means of non-linear numerical integration and optimization techniques in order to obtain further insight into the mechanism of this reaction and the source of its complexity.

Experimental

Sperm whale myoglobin type III was purchased from Sigma. The solid myoglobin was dissolved in 0.05 M tris(hydroxymethyl)amino methane buffer pH 8.0, and, in order to ensure that all the myoglobin was in the ferric state, a five fold excess of K$_4$Fe(CN)$_6$ was added. The excess Fe(CN)$_6^{3-}$ and Fe(CN)$_5^{2-}$ was then removed by passage through a G-25 Sephadex column. The concentrations of myoglobin solutions were measured from the absorbance of the deoxy Fe(H) form at 556 nm which has a pH independent extinction coefficient of 11.8 mM$^{-1}$ cm$^{-1}$ [17] (the deoxy Fe(H) form is obtained by reduction of Mb(III) with trace amounts of sodium dithionite). Bovine liver catalase was purchased from Boehringer Mannheim and the concentration of catalase solutions calculated using $e_{405} = 340$ mM$^{-1}$ cm$^{-1}$ [18]. Hydrogen peroxide was purchased from BDH Chemicals Ltd.; before each experiment a stock solution of approx. 0.1 M was standardized against KMnO$_4$ using the method of Allen [19] and then diluted to the desired concentration. All reagents were of analytical grade.

Fast kinetics were observed using a single beam Durrum-Gibson stopped-flow spectrophotometer. Slow absorbance, charges and absorption spectra were recorded on a Cary Model 212 spectrophotometer.

The experimental data were digitized by the method of Clore and Chance [2]. The overall standard deviation (S.D.) of the data was then plotted against the relative error of the value of the parameter. For values of S.D. less than 0.2, S.D. = $\Delta x/x$, where $x$ is the range of curve i. A value of $\bar{C}$ significantly greater than 1.0 (the expected root mean square value of $\bar{C}$ if the residuals for each curve were all independent random variables of zero mean and the same variance) indicates that the departures between calculated and observed values are systematic [2]. A quantitative measure of how accurately an optimized parameter has been determined is given by the standard deviation of its natural logarithm (S.D.$\ln$). For values of S.D.$\ln$ < 0.2, S.D.$\ln$ = $\Delta x/x$, the relative error of the value of the parameter, for larger values of S.D.$\ln$, up to 1 in magnitude, the parameter value is determined to within a factor $e^\pm 2.72$, and so its order of magnitude is known; significantly larger values of S.D.$\ln$ show that the observations are inadequate to determine the parameter.

In order to analyse complex kinetic data, it is essential to have a set of strict quantitative criteria on which to base one's choice of model. Such criteria have been developed by Clore & Chance [2] and consist of the following triple requirement: an S.D. within the standard error of the data, good determination of the optimized parameters and a random distribution of residuals. Thus for a given set of data, although there may be many models with an S.D. within the standard error of the data, models with too many degrees of freedom will fail such an analysis because of under-determination, and models with too few degrees of freedom will fail such an analysis as a result of the introduction of systematic errors in the distribution of residuals.

Results

In Figs. 1 and 2 a series of progress curves for the reaction of Mb(III) with H$_2$O$_2$, monitored at 408 nm, are shown under conditions where the molar ratio [H$_2$O$_2$]/[Mb]$_{total}$ lies in the range 0.3 to 4. The conformity of the data to the kinetic schemes given in Table I was tested by fitting the numerical solution of the ordinary differential equations derived for each scheme to the six progress curves in Figs. 1 and 2.
TABLE I. Schemes for the Reaction of Mb(III) with H₂O₂ together with their Overall Standard Deviations of the Fits and Mean Absolute Correlation Indices to the Data in Figs. 1 and 2.

Schemes B and C are analogous to the schemes for the reactions of horseradish peroxidase [4] and catalase [6] with hydrogen peroxide respectively. The mean absolute correlation index, \( C \), is a measure of the distribution of residuals and is defined by Eqn. (6). For \( C < 1.0 \), the distribution of residuals is random; for \( C >> 1.0 \), the deviations between the computed and observed progress curves are systematic [2]. The overall standard error of the data in Figs. 1 and 2 is 1.5 ± 0.2% with a 99% confidence interval of 1.1–1.9%.

<table>
<thead>
<tr>
<th>Scheme</th>
<th>S.D. (%)</th>
<th>( C )</th>
</tr>
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<tbody>
<tr>
<td>A. ( \text{Mb}(\text{III}) + \text{H}<em>2\text{O}<em>2 \xrightarrow{k</em>{+1}} X ) ( \xrightarrow{k</em>{+2}} R ) ( \xrightarrow{k_{+3}} \text{Mb}(\text{III}) )</td>
<td>1.2</td>
<td>0.98</td>
</tr>
<tr>
<td>B.(^a) ( \text{Mb}(\text{III}) + \text{H}<em>2\text{O}<em>2 \xrightarrow{k</em>{+1}} R \xrightarrow{k</em>{+2}} \text{Mb}(\text{III}) )</td>
<td>4.8</td>
<td>3.2</td>
</tr>
<tr>
<td>C.(^a) ( \text{Mb}(\text{III}) + \text{H}<em>2\text{O}<em>2 \xrightarrow{k</em>{+1}} R \xrightarrow{k</em>{-1}} \text{Mb}(\text{III}) )</td>
<td>5.4</td>
<td>3.3</td>
</tr>
<tr>
<td>D.(^a) ( \text{Mb}(\text{III}) + \text{H}<em>2\text{O}<em>2 \xrightarrow{k</em>{+1}} R \xrightarrow{k</em>{+2}} \text{Mb}(\text{III}) ) ( \xrightarrow{k_{+3}} Y )</td>
<td>5.3</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\(^a\)The addition of an extra first order step such that \( \text{Mb}(\text{III}) \rightarrow \text{R} \rightarrow \text{Mb}(\text{III}) \) is replaced by \( \text{Mb}(\text{III}) \rightarrow \text{R} \rightarrow \text{Mb}(\text{III}) \) does not improve the S.D.'s of the fits of schemes B, C and D, and results in poor determination of some of the optimized parameters (S.D.m >> 1). \(^b\)The addition of a minor species \( E^* \) either independent of \( E \) or in equilibrium with \( E \), and reacting with \( \text{H}_2\text{O}_2 \) by an identical mechanism to \( E \) but at a different rate, does not improve the S.D. of the fit of scheme B, and results in poor determination of some of the optimized parameters (S.D.m >> 1).

Fig. 1. Comparison of the observed (●) time courses at 408 nm for the reaction of Mb(II) with approximately stoichiometric concentrations of H₂O₂ with the best fit computed time courses for schemes A (——) and B (····). 8.49 \( \mu \text{M} \) (final concentration) \( \text{Mb}(\text{III}) \) was dissolved in 0.05 \( M \) tris(hydroxymethyl)amino methane buffer pH 8.0 at 20 °C and reacted with (a) 2.78 \( \mu \text{M} \), (b) 5.55 \( \mu \text{M} \), (c) 11.1 \( \mu \text{M} \), (d) 20.0 \( \mu \text{M} \), and (e) 35.1 \( \mu \text{M} \) (final concentrations) H₂O₂.

Fig. 2. Comparison of the observed (●) time course at 408 nm for the reaction of Mb(III) with an approximately stoichiometric concentration of H₂O₂ with the best fit time courses for schemes A (——) and B (····) showing the decay phase of the reaction. 8.49 \( \mu \text{M} \) (final concentration) \( \text{Mb}(\text{III}) \) dissolved in 0.05 \( M \) tris(hydroxymethyl)amino methane buffer pH 8.0 at 20 °C, was reacted with 20.0 \( \mu \text{M} \) (final concentration) H₂O₂.
TABLE II. Optimized Values of the Parameters for Scheme A together with Their S.D.1, and 5–95% Confidence Limits.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dimensions</th>
<th>Value</th>
<th>S.D.1</th>
<th>Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5%</td>
</tr>
<tr>
<td>$k_{+1}$</td>
<td>$M^{-1}\ s^{-1}$</td>
<td>$3.53 \times 10^2$</td>
<td>0.0163</td>
<td>$3.43 \times 10^2$</td>
</tr>
<tr>
<td>$k_{-1}$</td>
<td>$M^{-1}\ s^{-1}$</td>
<td>$3.40 \times 10^3$</td>
<td>0.0474</td>
<td>$3.14 \times 10^3$</td>
</tr>
<tr>
<td>$k_{+2}$</td>
<td>$s^{-1}$</td>
<td>$9.60 \times 10^{-5}$</td>
<td>0.0139</td>
<td>$9.38 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\Delta_{408}^{MB(III)}$</td>
<td>$M^{-1}\ cm^{-1}$</td>
<td>$1.04 \times 10^5$</td>
<td>0.0307</td>
<td>$0.98 \times 10^4$</td>
</tr>
<tr>
<td>$\Delta_{408}^{MB(III)-X}$</td>
<td>$M^{-1}\ cm^{-1}$</td>
<td>$7.19 \times 10^4$</td>
<td>0.0155</td>
<td>$7.01 \times 10^4$</td>
</tr>
</tbody>
</table>

2 simultaneously by means of non-linear optimization of the unknown parameters (i.e. rate constants and difference extinction coefficients). Only scheme A satisfied the triple requirement ([2], see Experimental section) of a S.D. within the standard error of the data (i.e. < 1.5%), good determination of the optimized parameters and a random distribution of residuals. The other kinetic schemes all failed on two counts: S.D. s greater than the standard error of the data and systematic errors in the distribution of residuals. The overall S.D. of the fit and the mean absolute correlation index (a measure of the nature of the distribution of residuals) for each scheme are given in Table I. The comparison of the experimental and computed progress curves in Figs. 1 and 2, systematic errors between the observed and computed curves for scheme B are seen. From Fig. 3 it can be seen that the dependence of the normalized amplitude of the reaction (defined as the ratio of the maximum absorbance change produced in the reaction, $\Delta A_{max}^{MB(III)-R}$, to the absorbance change produced by the complete conversion of Mb(III) to species R, $\Delta A_{MB(III)-R}$) on the ratio $[H_2O_2]/[Mb]_{total}$ is shown in Fig. 3. As in the case of the progress curves in Figs. 1 and 2, systematic errors between the observed and computed curves for scheme B are seen. From Fig. 3 it can be seen that the dependence of the normalized amplitude of the reaction ($\Delta A_{max}^{MB}/\Delta A_{MB(III)-R}$) on the ratio $[H_2O_2]/[Mb]_{total}$ is non-linear and approaches a maximum of 1 when $[H_2O_2]/[Mb]_{total} \approx 4$. If the reaction proceeded by a single step irreversible second order mechanism (i.e. Mb(III) + $H_2O_2 \rightarrow R$) then the curve would be a straight line with a slope of 1 up to $[H_2O_2]/[Mb]_{total} = 1$, and thereafter a horizontal line.

The computed dependence for scheme A of $[R]_{max}/[Mb]_{total}$ and $[X]_{max}/[Mb]_{total}$ on the ratio $[H_2O_2]/[Mb]_{total}$ is also shown in Fig. 3. The curve for $[R]_{max}/[Mb]_{total}$ is sigmoid and reaches a maximum of 1 when $[H_2O_2]/[Mb]_{total} \approx 4$. The curve for $[X]_{max}/[Mb]_{total}$ reaches a maximum of 0.08 when $[H_2O_2]/[Mb]_{total} \approx 0.4$, and the value of $[X]_{max}/[Mb]_{total}$ remains unchanged for all higher values of $[H_2O_2]/[Mb]_{total}$.

The optimized values of the unknown parameters for scheme B are given in Table III. The comparison of the experimental and computed progress curves for scheme B is shown in Figs. 1 and 2. The best fit curves for scheme B (which gave the second best fit after scheme A) are also shown in Figs. 1 and 2, and clearly demonstrate the presence of systematic errors between the observed and computed curves. The optimized values of the unknown parameters for scheme B are given in Table III.
The reaction of Mb(III) with H$_2$O$_2$ yield a series of spectra with a single set of isosbestic points. This is confirmed in Fig. 5 where a single set of isosbestic points at 341 and 417 nm is observed in the Soret region.

(3) Under conditions of a large molar excess of H$_2$O$_2$ over myoglobin the progress curves of the reaction are monophasic and the kinetics are pseudo-first order up to high H$_2$O$_2$ concentrations (Fig. 6). The apparent second order rate constant corresponds to $k_{+1}$ and the data in Fig. 7 has a value of $3.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ which lies within the confidence limits of the value of $k_{+1}$ found by non-linear optimization (Table II).

(4) The value of $k_{+3}$ can only be found under conditions where the concentrations of H$_2$O$_2$ and myoglobin are approximately stoichiometric by non-linear optimization procedures.

As species R is immediately reformed from Mb(III) via species X in the presence of H$_2$O$_2$, the rate constant for the reaction

$$R \xrightarrow{k_{+3}} \text{Mb(III)}$$

cannot be estimated from a semi-logarithmic plot of the decay phase of the reaction (see Fig. 2). Such a plot would yield a value of $k_{+3}$ that was significantly underestimated. Thus, under these conditions the value of $k_{+3}$ can only be determined by non-linear optimization. An accurate value of $k_{+3}$, however, may be obtained from a semi-logarithmic plot of the decay phase by removing all the remaining H$_2$O$_2$ by the addition of trace amounts of catalase ([catalase] / [Mb]$_{\text{total}} < 1/150$) after all the Mb(III) has been converted into species R. Catalase catalyses the overall reaction

$$2\text{H}_2\text{O}_2 \xrightarrow{k} 2\text{H}_2\text{O} + \text{O}_2$$

with an apparent second order rate constant $k = 4 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ [6] so that all the H$_2$O$_2$ present is removed rapidly (<5 min). Thus the only reaction observed approximately 5 min after the addition of catalase is the spontaneous reduction of species R to Mb(III) which proceeds by a first order process with a rate constant of $9.8 \times 10^{-9} \text{ s}^{-1}$ determined from the semi-logarithmic plot in Fig. 7. This value of $k_{+3}$ lies within the confidence limits of the value of $k_{+3}$ obtained by non-linear optimization (Table II).

Discussion

The state of the iron atom in species R has been studied by Mössbauer [7], resonance Raman [8] and magnetic susceptibility [9] studies, all of which provide strong evidence for a low spin Fe(IV) state. Although the nature of the ligand in the sixth coordination position of the iron atom is unknown, several
of its features may be deduced. O₂ is evolved during the reaction of Mb(III) with H₂O₂ and its evolution exhibits an appreciable lag phase in relation to the formation of species R [15] which indicates that O₂ is evolved during the spontaneous first order reduction of species R to Mb(III). This suggests that the ligand in the sixth coordination position contains two atoms of oxygen.

The observation of an EPR detectable transient free radical species at g ~ during the reaction of Mb(III) with H₂O₂ reaching a maximum spin concentration of approximately 10% of the total myoglobin concentration [14] may be correlated to species X. Given that the OH radical would be
Reaction of Mb(III) with H$_2$O$_2$

[Graph: Semi-logarithmic plot of the spontaneous reduction of species R to Mb(III) following the addition of trace amounts of catalase to remove all remaining H$_2$O$_2$. 5.8 \mu M (final concentration) Mb(III) dissolved in 0.05 M tris(hydroxymethyl) amino methane buffer pH 8.0 at 20 °C was allowed to react with 92 n&f (final concentration) H$_2$O$_2$ until the absorbance change at 408 nm reached a maximum and all the myoglobin was in the form of species R (~10 min). 0.036 \mu M (final concentration) catalase was then added and the subsequent reduction of species R to Mb(III) monitored at 408 nm.]

expected to be undetectable owing to its very short lifetime at room temperature (the reaction OH$^{-}$ + OH$^{+}$ → H$_2$O$_2$ proceeding with a second order rate constant of 5.5 × 10$^9$ M$^{-1}$ s$^{-1}$ [22]), it seems likely that the free radical is either a protein cation radical as in cytochrome c peroxidase [5] or a porphyrin n-cation radical. In the latter case, unlike in compound I of horseradish peroxidase [4], the electron localized on the porphyrin would not be coupled through exchange interactions with spin localized on the haem iron, thereby accounting for the presence of a g ~ 2 EPR signal. The spectral similarity in the Soret region of species X and R deduced from Fig. 5 (see Results section) suggests that the iron atom in both species is in the same formal valence state, namely Fe(IV).

The observation in unbuffered solutions that the formation of species R is accompanied by the release of one proton [23] indicates that ionizing groups in the active site are involved in the reaction of Mb(III) with H$_2$O$_2$.

On the basis of our kinetic analysis and the above evidence, we suggest the following mechanism for the reaction of Mb(III) with H$_2$O$_2$ at pH 8.0. The first step is a two electron transfer reaction resulting in the oxidation of Mb(III) to species X:

\[
\begin{align*}
B_{1}^{-} & \quad \text{Mb(III)} \\
Fe^{3+} & \quad +H_{2}O_{2} \quad \text{3.53} \times 10^{9} \text{M}^{-1} \text{s}^{-1} \\
\text{Z} & \quad \text{species X} \\
B_{2}^{-} & \quad H_{2}O \\
\end{align*}
\]

where Z represents either an amino acid in the active site or the porphyrin. Z$^{\cdot}$ is the cation free radical produced by the oxidation of Z, and B$_1$H and B$_2$H are ionizing groups in the active site. The second step is a one electron reduction of species X by H$_2$O$_2$, resulting in the formation of species R:

\[
\begin{align*}
B_{1}^{-} & \quad k_{+1} \\
Fe^{4+}\cdot OH^{-} & \quad 3.40 \times 10^{4} \text{M}^{-1} \text{s}^{-1} \\
Z^{\cdot} & \quad +H_{2}O_{2} \quad +H_{2}O + H^{+} \\
B_{2}^{-} & \quad \text{species R} \\
\end{align*}
\]

As Fe$^{4+}\cdot$OOH is of integral spin, no EPR signals are seen in species R [24]. It should be noted that the configuration Fe$^{4+}\cdot$O$_2^{-}$ for species R can be excluded as it would be expected to exhibit an EPR signal. In the third step, species R undergoes an internal oxidation-reduction reaction resulting in the reduction of Fe$^{4+}$ to Fe$^{3+}$ and the oxidation of the liganded -OOH to O$_2$:

\[
\begin{align*}
B_{1}^{-} & \quad k_{+1} \\
Fe^{4+}\cdot OH^{-} & \quad 9.60 \times 10^{4} \text{M}^{-1} \text{s}^{-1} \\
Z & \quad +H^{+} \\
B_{2}^{-} & \quad \text{species R} \\
\end{align*}
\]

\[
\begin{align*}
B_{1}^{-} & \quad k_{-1} \\
Fe^{4+}\cdot OH^{-} & \quad 9.60 \times 10^{4} \text{M}^{-1} \text{s}^{-1} \\
Z & \quad +O_{2} \\
B_{2}^{-} & \quad \text{species R} \\
\end{align*}
\]

The overall stoichiometry of the reaction of Mb(III) with H$_2$O$_2$ corresponds to that of the classical scheme for the reaction of ferric catalase with H$_2$O$_2$ given by [25]:

\[
\text{Cat(Fe}^{3+}) + H_{2}O_{2} \xrightleftharpoons[k_{+1}]{k_{-1}} \text{compound I (Z$^{\cdot}$Fe$^{4+}$)} \\
\text{compound I + H}_{2}O_{2} \xrightarrow{k_{+2}} \text{Cat + 2H}_{2}O + O_{2} \\
\]

Thus, it is useful to compare and contrast the two systems.

The first step in the reactions of both Mb(III) and ferric catalase with H$_2$O$_2$ appears to involve a direct two electron transfer resulting in the formation of an intermediate two oxidizing equivalents above Mb(III) and ferric catalase: species X in the case of the Mb(III) reaction and compound I [6] in the catalase reaction. The second order rate constant for the formation of species X is smaller than that for the formation of compound I by a factor of about 10$^5$ (cf.
Table II and ref. 25). This difference may be due to steric factors imposed by the apoprotein limiting access of \( \text{H}_2\text{O}_2 \) to the iron atom. This view is supported by the observation that the rate constants for the formation of complexes between Mb(III) and small ligands are in general much smaller than those for the corresponding reactions with ferric catalase [26].

In the second stage of the catalase reaction \( \text{O}_2 \) is evolved and ferric catalase regenerated in a process that appears to involve only a single step (i.e. an apparent direct two electron reduction of compound I) as far as can be determined from kinetic measurements [6, 10, 25]. If a species corresponding to species R (i.e. a compound II-like species) is an intermediate in the catalase reaction, then, given that the overall reaction is second order up to a concentration of \( 1 \text{ M} \text{H}_2\text{O}_2 \) and \( k_{\text{app}}^{\text{II}} \sim 2 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \) [25], the rate constant (\( k_{s+} \)) for its breakdown must be greater than \( 10^8 \text{ s}^{-1} \). Thus, if the reactions of ferric catalase and Mb(III) with \( \text{H}_2\text{O}_2 \) proceed by similar mechanisms, this would give a 10\(^{12}\) fold difference in the stability of the respective R species (as \( k_{s+} \sim 10^{-4} \text{ s}^{-1} \) in the Mb(III) system). On the other hand if the catalase reaction does indeed proceed by a mechanism involving a direct two electron reduction of compound I by \( \text{H}_2\text{O}_2 \), then the importance of the apoprotein in transforming the reactivity of the haem group in catalase away from that in the 'model' Mb(III) system is even further emphasized.

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