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The Kinetics of the Reactions of Low Spin Ferric Haem Undecapeptide with Hydrogen Peroxide

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The kinetics of the reaction of low spin ferric haem undecapeptide (HUP^{III}) with H_2O_2 at pH 7.0 and 10.4 at 22 °C have been investigated by stopped flow spectroscopy at 398 nm and analysed by non-linear numerical integration and optimization techniques. At pH 7.0, the only reaction scheme which is found to satisfy the triple requirement of a standard deviation within the standard error of the data, good determination of the optimized parameters and a random distribution of residuals is a four-step mechanism stated as follows:

$$HUP_C^{III} + H_2O_2 \rightarrow I_C \rightarrow II_C \rightarrow III_C,$$

$$III_C + H_2O_2 \rightarrow IV_C$$

At pH 10.4 the only scheme which is found to satisfy the above triple requirement is a condensed version of the mechanism at pH 7.0 and can be represented as:

$$HUP_D^{III} + H_2O_2 \rightarrow I_D \rightarrow III_D,$$

$$III_D + H_2O_2 \rightarrow IV_D$$

The rate constants for each step and the difference extinction coefficient at 398 nm of HUP^{III} minus each intermediate species have been determined. The spectral characteristics of the intermediate species in the Soret region have been obtained by rapid wavelength scanning stopped flow optical spectroscopy. The possible nature of the intermediate species are discussed in relation to data in the literature on the reactions of aquo Fe(III) myoglobin, catalase and horseradish peroxidase with H_2O_2.

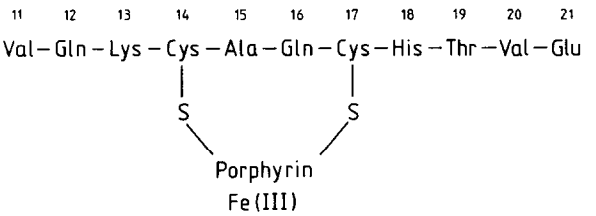

Fig. 1. The amino acid sequence of HUP^{III}. The numbering of the residues refers to native cytochrome c (from ref. 11).

Introduction

As part of our continuing study on the factors determining the reactivity of the haem group in simple redox reactions [1–3], we have examined in detail the reactions of low spin ferric haem undecapeptide (HUP^{III}) with H_2O_2.

The manner in which the apoprotein affects the behaviour of the haem is of considerable interest. Recently we examined in detail the kinetics of the reaction of aquo Fe(III) myoglobin with H_2O_2 and compared it to the reactions of horseradish peroxidase and catalase with H_2O_2 [3]. To further extend our knowledge of the reactions of haem proteins with H_2O_2 it would be important to examine the reaction of ferric haem with H_2O_2 with the haem freed from the constraints imposed by the protein (namely, a hydrophobic environment and a fixed distribution of axial ligands). Unfortunately 'naked'

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TABLE I. Axial Coordination and Spin State of the Central Iron Atom of the Four pH Dependence Species of HUP^{III}. The pH range over which each species is present in greater than 90% of the total HUP concentration together with the pK's are also given (from ref. 8).

Species	pKa	pH range over which each species is present in 90% of total HUP concn.[a]	Axial ligands to the central iron atom		Spin state[b]
			5th position	6th position	
HUP_A^{III}	3.4	<2.2	H_2O ?	H_2O ?	high spin
HUP_B^{III}	5.8	3.8–4.8	His_{18} (intramolecular)	H_2O	high spin
HUP_C^{III}	7.6	6.8–9.0	His_{18} (intramolecular)	αNH_2 of val_{11} or ϵNH_2 of lys_{13} (intermolecular)	low spin
HUP_D^{III}		<10.0	His_{18} (intramolecular)	ϵNH_2 of lys_{13} or αNH_2 of val_{11} (intermolecular)	low spin

[a]From pH titrations monitored optically in the Soret region [8]. [b]The high spin–low spin transition has a pK of *ca.* 6 as determined by magnetic susceptibility measurements [7]. This corresponds to the optical transition with a pK of 5.8.

haem is unsuitable for study due to its instability and tendency to form dimers in aqueous solution. An alternative approach, therefore, is to study haem peptides produced by the enzymatic degradation of cytochrome c. The best characterised and most extensively studied of these is HUP^{III} [4–11], the amino acid sequence of which is shown in Fig. 1. HUP^{III} is stable over a wide range of pH values, and maintains the haem in an aqueous environment unlike the hydrophobic one within a protein.

The axial ligand environment of HUP^{III} is pH dependent. Four pH dependent species have been demonstrated [8]. The pK's for their interconversion, the pH ranges over which each species is present in concentrations greater than 90% of the total HUP concentration, the suggested axial ligands to the central iron atom and the spin state of the iron atom are summarised in Table I. In the present paper we have examined the kinetics of the reaction of the two low spin HUP^{III} species with H_2O_2: species HUP_C^{III} at pH 7.0 and species HUP_D^{III} at pH 10.4.

Experimental

HUP^{III} was prepared from bovine cytochrome c (purchased from Sigma) as described by Peterson *et al.* [11]. HUP^{III} prepared by this method has a high degree of purity and good reproducibility from preparation to preparation as determined by amino acid analysis, isoelectric focussing and Mössbauer spectroscopy. A concentrated stock solution (300 μM) was prepared by dissolving lyophilised HUP^{III} in distilled water, and stored at −5 °C. Solutions of HUP^{III} for stopped flow experiments were prepared by diluting the concentrated stock solution in the appropriate buffer (0.1 *M* sodium phosphate pH 7.0 or 0.1 *M* sodium borate pH 10.4). H_2O_2 was purchased from BDH Chemicals Ltd.; before each experiment a stock solution of approx. 0.1 *M* was standardised against $KMnO_4$ using the method of Allen [12] and then diluted to the desired concentration. All reagents were of the highest purity commercially available.

Kinetic measurements were carried out at 22 °C using a single beam Durrum-Gibson stopped flow spectrophotometer with a dead time of 2.7 ms and a path length of 1.7 cm. Rapid wavelength scanning stopped flow spectroscopy was carried out as described previously [1, 13], with the exception that spectra were recorded on videotape at a rate of 50 frames per sec. As each spectrum was recorded at a rate of 1 spectrum per ms, each recorded frame on videotape contained the average of approximately 20 individual spectra.

In order to exclude the possibility that HUP^{III} exists as an equilibrium mixture of two or more species (*e.g.* monomers and polymers of differing molecular weights) we carried out a series of control experiments at each pH using different concentrations of HUP^{III}. In the concentration range (1–5 μM HUP^{III}) over which the experiments reported in this paper were carried out, no difference in the kinetics of the reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4 could be detected, thus indicating that at both pH values in this concentration range, HUP^{III} is present as a single species.

The experimental data were digitised by the method of Clore and Chance [14]. The overall standard error for the kinetic data at each pH was 2.0 ± 0.2%. The kinetic data were analysed by means of non-linear stiff numerical integration and optimisation techniques as described previously [13, 14, 15].

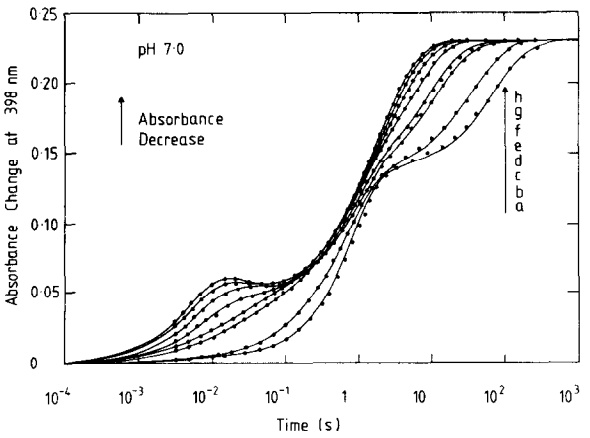

Fig. 2. Observed and computed kinetics of the reaction of HUP^{III} with H_2O_2 at pH 7.0 monitored at 398 nm. Symbols: •, observed time courses; ———, best fit computed time curves for the scheme at pH 7.0 shown in Table II. Experimental conditions: 1.63 μM (final concentration in the mixing chamber) HUP^{III} in 0.1 M sodium phosphate buffer pH 7.0 at 22 °C (syringe 1) was reacted with H_2O_2 dissolved in the same buffer (syringe 2). The final concentration of H_2O_2 in the reaction chamber were: (a) 1.29 mM, (b) 2.58 mM, (c) 7.79 mM, (d) 10.3 mM, (e) 16.5 mM, (f) 22.8 mM, (g) 28.8 mM, and (h) 33.1 mM. Path length 1.7 cm.

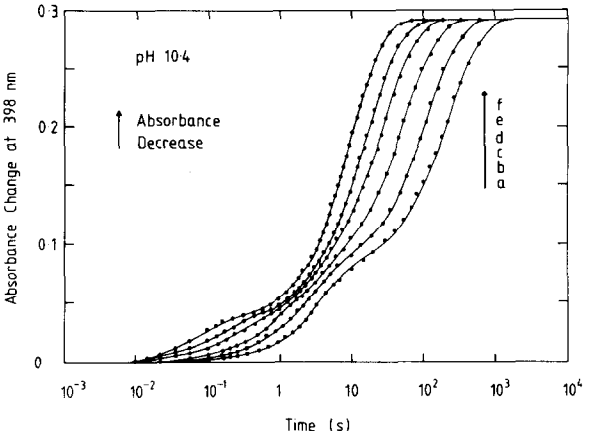

Fig. 3. Observed and computed kinetics of the reaction of HUP^{III} with H_2O_2 at pH 10.4 monitored at 398 nm. Symbols: •, observed time course, ———, best fit computed time courses for the scheme at pH 10.4 shown in Table II. Experimental conditions: 1.63 μM (final concentration in the mixing chamber) HUP^{III} in 0.1 M sodium borate buffer pH 10.4 at 22 °C (syringe 1) was reacted with H_2O_2 dissolved in the same buffer (syringe 2). The final concentrations of H_2O_2 in the mixing chamber were: (a) 2.79 mM, (b) 5.58 mM, (c) 11.5 mM, (d) 22.3 mM, (e) 35.9 mM and (f) 71.8 mM. Path length 1.7 cm.

Results and Discussion

Kinetic analysis of the reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4

The progress curves for the reaction of HUP^{III} with H_2O_2 monitored at 398 nm and 22 °C at pH 7.0 and 10.4 are shown in Figs. 2 and 3 respectively.

Our choice of the minimal model required to fit the experimental data was based on the triple requirement of Clore and Chance [14, 15]: (1) a S.D. within the standard error of the data, (2) a random distribution of residuals and (3) good determination of the optimised parameters. These three criteria provide a rigorous framework on which to base one's choice of model. Thus for a given set of data, although there may be many models with a S.D. within the standard error of the data, models with too many degrees of freedom will fail such an analysis because of underdetermination, 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. Indeed, it is often the case that only a single model will satisfy all three criteria [2, 3, 14–17].

At both pH values the progress curves at all concentrations were fitted simultaneously to a given kinetic model (*i.e.* eight and six concentrations of H_2O_2 at pH 7.0 and 10.4 respectively).

TABLE II. The Minimal Schemes for the Reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4 which Satisfy the Triple Requirement of a S.D. within the Standard Error of the Data, a Random Distribution of Residuals and Good Determination of the Optimised Parameters. The overall S.D.'s of the fits and mean absolute correlation indices to the data in Figs. 2 and 3 are given. The overall standard error of the data at each pH is 2.0 ± 0.3%. The mean absolute correlation index, $\bar{C}$, is a measure of the distribution of residuals (see ref. 14 for definition); for $\bar{C} \lesssim 1$, the distribution of residuals is random; for $\bar{C} \gg 1$, the deviations between the computed and observed progress curves are systematic [14].

pH	Scheme	SD %	$\bar{C}$
7.0	$HUP^{III}_C + H_2O_2 \xrightarrow{k_1} I_C$ $I_C \xrightarrow{k_2} II_C \xrightarrow{k_3} III_C$ $III_C + H_2O_2 \xrightarrow{k_4} IV_C$	2.20	1.25
10.4	$HUP^{III}_D + H_2O_2 \xrightarrow{k_1} I_D$ $I_D \xrightarrow{k_2} III_D$ $III_D + H_2O_2 \xrightarrow{k_4} IV_D$	2.09	1.62

TABLE III. Optimized Values of the Parameters for the Reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4. The standard deviation of the natural logarithm ($S.D._{ln}$) of the optimized parameters are given in parentheses. For $S.D._{ln} < 0.2$, $S.D._{ln} \sim \Delta x/x$ where $\Delta x/x$ is the fractional error; for $S.D._{ln} = 1$, the parameter value is determined to within a factor $e \sim 2.72$; for $S.D._{ln} \gg 1$, the parameter is ill determined.

Parameters	pH (HUP^{III})	
	7.0 (HUP^{III}_C)	10.4 (HUP^{III}_D)
k_1 (M^{-1} s^{-1})	2.15×10^3 (0.0752)	1.73×10^2 (0.139)
k_2 (s^{-1})	1.47×10^2 (0.228)	4.54×10^{-1} (0.0721)
k_3 (s^{-1})	1.24 (0.0522)	–
k_4 (M^{-1} s^{-1})	1.11×10^1 (0.0537)	1.47 (0.0228)
$\Delta\epsilon_{398}$(HUP^{III}–I) (M^{-1} cm^{-1})	6.49×10^4 (0.132)	1.11×10^4 (0.0879)
$\Delta\epsilon_{398}$(HUP^{III}–II) (M^{-1} cm^{-1})	1.88×10^4 (0.0279)	2.81×10^4 (0.0210)
$\Delta\epsilon_{398}$(HUP^{III}–III) (M^{-1} cm^{-1})	5.24×10^4 (0.0142)	–
$\Delta\epsilon_{398}$(HUP^{III})IV) (M^{-1} cm^{-1})	8.60×10^4 (0.00511)	1.04×10^5 (0.00433)

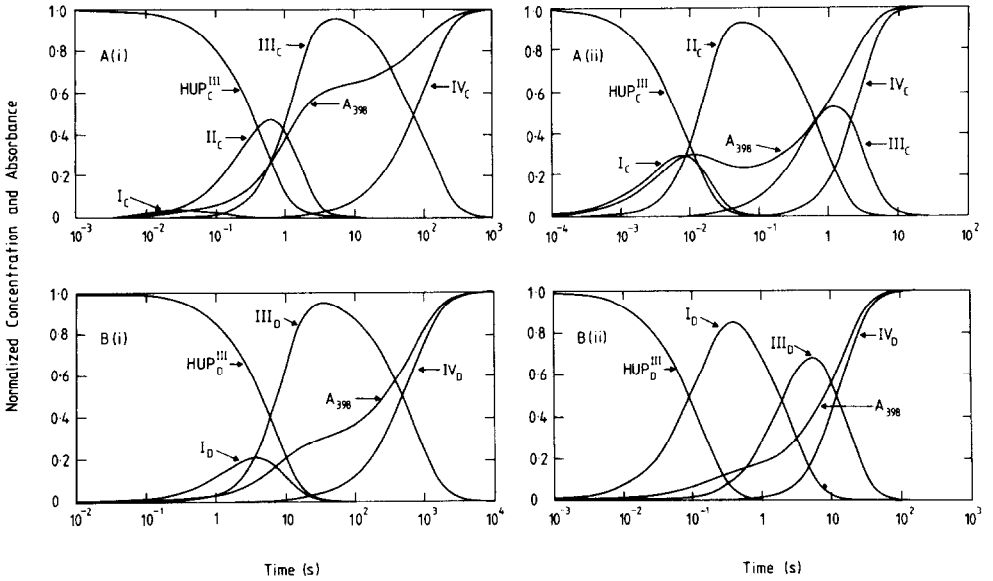

Fig. 4. Computed time courses of the concentrations of the intermediate species and absorbance changes at 398 nm in the reactions of HUP^{III} at pH 7.0 (A), and 10.4 (B) for the schemes shown in Table II. Initial conditions: 2 μM HUP^{III}, (i) 1 mM and (ii) 50 mM H_2O_2. The values of the rate constants and difference extinction coefficients (HUP^{III} minus intermediate species) at each pH are given in Table III.

Four distinct phases are seen in the kinetics at pH 7.0 (Fig. 2) and three phases at pH 10.4 (Fig. 3). The minimal scheme at each pH which satisfies the triple requirement of Clore and Chance [14, 15] is given in Table II. The overall S.D. of the fit and mean absolute correlation index for each scheme is given in Table II, the values of the optimised parameters together with the standard deviations of their natural logarithms ($S.D._{ln}$) are given in Table III. The best fit computed curves for the reactions at pH 7.0 and 10.4 are shown in Figs. 2 and 3 respectively, and the computed time courses of the concentrations of the intermediate species at both pH values are shown in Fig. 4 at two concentrations of H_2O_2 (1 mM and 50 mM).

Schemes involving fewer steps fail to fit the data at all three pH values on the basis of criteria (1) and (2) stated above, and more complicated schemes involving more steps fail on account of criteria (3). It should also be noted that schemes, involving two

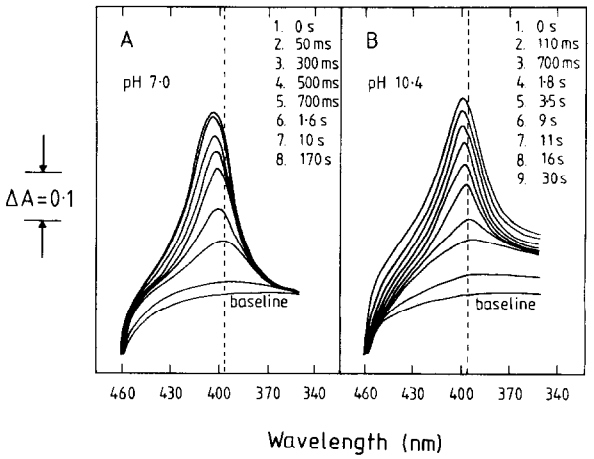

Fig. 5. Time resolved spectra in the Soret region obtained during the reactions of HUP^{III} with H_2O_2 at pH 7.0 (A) and 10.4 (B) by rapid wavelength scanning stopped flow optical spectroscopy. Experimental conditions: HUP^{III} in (A) 0.1 *M* sodium phosphate buffer pH 7.0 and (B) 0.1 *M* sodium borate buffer pH 10.4 (syringe 1) was reacted with H_2O_2 dissolved in the corresponding buffers (syringe 2) at 22 °C. The final concentration of HUP^{III} in the mixing chamber was 3.8 μM. The final concentrations of H_2O_2 in the mixing chamber were: 1 m*M* (A) and 50 m*M* (B). Path length 1.0 cm.

species of HUP^{III} in equilibrium also fail to fit the data at both pH values on the basis of all three criteria.

The reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4 are both characterised by two second order steps, namely the first and last steps in the reaction. Schemes in which only the first step is second order, or in which the second of the second order steps is not placed at the last step of the reaction, fail to fit the data on account of criteria (1) and (2).

From a comparison of the values of the rate constants at the two pH values, it can be seen that the rate constants for each step are significantly greater at pH 7.0 than at pH 10.4 by a factor ranging from one to two orders of magnitude, indicating that the kinetic properties of the low spin ferric haem towards H_2O_2 are dramatically affected by the nature of the ligand in the 6th axial position. It should be noted that at pH 7.0, species I_C is converted to species III_C via species II_C:

$$I_C \xrightarrow{k_2} II_C \xrightarrow{k_3} III_C$$

Species II_C is detectable at pH 7.0 because $k_2 > k_3$. At pH 10.4 however, the corresponding species II_D is not detectable, and we observe the apparent direct conversion of species I_D to species III_D:

$$I_D \xrightarrow{k_2} II_D$$

TABLE IV. Absorption Maxima in the Soret Region of the Intermediate Species Formed in the Reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4.

	pH 7.0 (HUP^{III}_C)	pH 10.4 (HUP^{III}_D)
HUP	403	400
I	[a]	398
II	400	–
III	398	397
IV	[b]	[b]

[a]The maximal concentration of species I_C at pH 7.0 is too low (<6% of the total concentration of HUP) to produce any significant change in the spectrum of native HUP. [b]The spectra of species IV_C and IV_D at pH 7.0 and 10.4 respectively are featureless and almost completely bleached.

Therefore, if species II_D is indeed formed at pH 10.4, k_3 must be very much greater than k_2 at this pH.

Rapid wavelength scanning stopped flow optical spectroscopy in the Soret region

In order to characterise the spectra of the intermediate species in the Soret region formed in the reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4 we have used rapid wavelength scanning stopped flow optical spectroscopy. Typical rapid wavelength scanning spectra are shown in Fig. 5. Several features are noteworthy:

1) No dramatic wavelength shifts are seen. However, there are small wavelength shifts which by correlating the spectra to the computed time courses of the intermediate species shown in Fig. 4 can be assigned to the various intermediate species (Table IV). Despite the observation that the wavelength shifts between the intermediate species are small, there are large differences in extinction coefficients between HUP^{III} and the intermediate species shown in Table III.

2) The spectra of species IV_C and IV_D, the end products of the reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4 respectively, are featureless and virtually completely bleached. This indicates that the last second order step at both pH values involves attack by H_2O_2 on the haem ring resulting in the destruction of the latter.

Nature of the intermediate species formed in the reactions of HUP^{III} with H_2O_2 at pH 7.0 and 10.4

The spectral data in the Soret region obtained by rapid wavelength scanning stopped flow spectroscopy, unfortunately do not enable one to assign the valence state of the iron atom in the intermediates formed in the reactions of HUP^{III} with H_2O_2. Nevertheless, by analogy with the reactions of aquo Fe(III) myoglobin, catalase and horseradish peroxidase with

H_2O_2 [3, 16–18], it seems likely that $X^{+\bullet}Fe^{4+}$ (where $X^{+\bullet}$ is either a protein cation or porphyrin-π cation radical) and Fe^{4+} species are formed.

The first step in the reactions of aquo Fe(III) myoglobin, catalase and horseradish peroxidase with H_2O_2 involve the two electron reduction of H_2O_2 [3, 18–20] coupled to the oxidation of Fe^{3+} to Fe^{4+} and X (where X is either a protein moiety or the porphyrin ring) to $X^{+\bullet}$. It therefore seems likely that the first step in the reactions of HUP^{III} with H_2O_2 at both pH 4.7 and 7.0 similarly involves a two electron reduction of H_2O_2 and can be represented as

$$X\cdot Fe^{3+} + H_2O_2 \xrightarrow{k_1} X^{+\bullet}\cdot Fe^{4+}\cdot O^{2-} + H_2O$$

HUP^{III}_C I_C

HUP^{III}_D I_C

The values of the rate constant for this step obtained with HUP^{III} (2150 M^{-1} s^{-1} at pH 7.0; 173 M^{-1} s^{-1} at pH 10.4) are four to five orders of magnitude smaller than those for the corresponding step in the reactions of catalase (~4 × 10^7 M^{-1} s^{-1} [21]) and horseradish peroxidase (~2 × 10^7 M^{-1} s^{-1} [22]) with H_2O_2. However, in the case of the reaction of aquo Fe(III) myoglobin with H_2O_2, the value of rate constant for this step (353 M^{-1} s^{-1}, is of the same order of magnitude as that for the reaction of HUP^{III} with H_2O_2 at pH 10.4 but an order of magnitude smaller than that at pH 7.0. This clearly demonstrates the role of the protein moiety in catalase and horseradish peroxidase in stabilising the transition state on the reaction pathway to the $X^{+\bullet}Fe^{4+}$ species by approximately 20–30 kJ mol^{-1} relative to the corresponding transition state in the reactions of both HUP^{III} and aquo Fe(III) myoglobin with H_2O_2.

Following the first step, the next two steps at pH 7.0 probably involve internal oxidation-reduction reactions. These could possibly be of the type:

$$X^{+\bullet}\cdot Fe^{4+}\cdot O^{2-} \xrightarrow{k_2} X\cdot Fe^{4+}\cdot O^{-} \xrightarrow{k_3} X^{+\bullet}\cdot Fe^{3+}\cdot O^{-}$$

or

$$X^{+\bullet}\cdot Fe^{4+}\cdot O^{2-} \xrightarrow{k_2} X^{+\bullet}\cdot Fe^{3+}\cdot O^{-} \xrightarrow{k_3} X\cdot Fe^{4+}\cdot O^{-}$$

I_C II_C III_C

At pH 10.4, the second step also probably involves an internal oxidation reduction of the following type:

$$X^{+\bullet}\cdot Fe^{4+}\cdot O^{2-} \xrightarrow{k_2} X\cdot Fe^{4+}\cdot O^{-}$$

I_D III_D

or

$$X^{+\bullet}\cdot Fe^{4+}\cdot O^{2-} \xrightarrow{k_2} X^{+\bullet}\cdot Fe^{3+}\cdot O^{-}$$

I_D III_D

The last step at both pH 7.0 and 10.4, involves a further attack by H_2O_2 resulting in the destruction of the haem, as demonstrated by the featureless and bleached nature of the spectrum of species IV_C and IV_D (Fig. 5). It seems likely that this process is not a single step process but rather a multi-step one, probably involving free radical formation, in which the rate constants for the subsequent steps are all significantly greater than k_4.

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