The Kinetics of Oxidation of Ferroperoxidase by Molecular Oxygen

A MODEL OF A TERMINAL OXIDASE

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1. The decay of oxyferroperoxidase to ferriperoxidase was studied by rapidly mixing solutions of ferroperoxidase with various amounts of oxygen and following the time-course of appearance of oxyferroperoxidase and its subsequent decay to ferriperoxidase by reaction with ferroperoxidase. 2. The scheme can be accommodated by

and

and occurs without detectable intermediates being observed in the millisecond time-range.

3. Analogue-computer simulation of the reaction is in agreement with the initial rate-limiting reaction being an intermolecular, not intramolecular, electron-donating process, and analysis of the data leads to quantitative values for the rate constants of the overall process. 4. The reaction of oxyferroperoxidase with ferroperoxidase is a model of a terminal oxidase, and the results are discussed in terms of the possible importance of this reaction in peroxidase function, and also in the light it throws on autoxidation of haem compounds.

Horseradish peroxidase is a monomeric protein containing a single protoporphyrin IX haem group. It has a curious duality of nature. Thus, whereas the absorption spectra of both ferric and ferrous derivatives emphasize its similarity to haemoglobin and myoglobin and its ligand-binding ability mirrors qualitatively that of the other two haem proteins, the most studied reactions are those in which the enzyme catalyses the oxidation of a variety of substrates by H_2O_2 , by putative mechanisms involving formal oxidation states of the iron of +5 and +4.

These two aspects of peroxidase reactivity lead to an apparent contradiction. Although peroxidase has the attributed function of a catalyst of oxidation by H_2O_2 , the finding by Wittenberg *et al.* (1967) that stoichiometric oxygen complexes of the ferrous species could be formed, which decayed without detectable intermediates to ferric peroxidase, suggest that this enzyme resembles a terminal oxidase in performing the four-electron oxidation in which the formal oxidation state of iron is +6.

This paper is concerned with an exploration of this reaction, both as to the light that it may shed on peroxidase reactivity and also as a convenient model of a

terminal oxidase. Solutions of ferroperoxidase were rapidly mixed with buffer solution containing various concentrations of oxygen and the time-course of the formation of oxyferroperoxidase and its subsequent decay to ferriperoxidase were followed. The reaction scheme constructed from an analysis of the data has been subjected to stringent testing by analogue computation and it is concluded that within the time-scale of observation oxyferroperoxidase is first generated and then reacts in the manner described above to produce ferriperoxidase without detectable intermediates being observed.

Experimental

Horseradish peroxidase (isoenzyme B; Shannon *et al.*, 1966) was purified as described by Phelps & Antonini (1969).

Solutions of peroxidase were made in 0.1 M-sodium phosphate buffer, pH 7.0, and were approx. 0.1 mM, as determined spectrophotometrically. To this solution in a 20 ml syringe was added 10 μl of catalase (0.3 mM; determined spectrophotometrically) and the solution was titrated with approx. 450 μl of 10 mM- $\text{Na}_2\text{S}_2\text{O}_4$ in 1 mM-NaOH, to effect complete reduction of ferriperoxidase and to remove dissolved oxygen.

The catalase was present to remove H_2O_2 formed by the action of $\text{Na}_2\text{S}_2\text{O}_4$, since failure to do so resulted in anomalous kinetics.

Oxygen solutions were prepared by mixing the air-saturated buffer (20°C and 1 atm) with degassed argon-equilibrated buffer solutions.

Oxyferroperoxidase was generated statically by adding 10mg of freshly reduced peroxidase in 0.5ml of the buffer to the top of a column (2.5 cm $\times$ 15 cm) of Sephadex G-25 (coarse grade) previously equilibrated with the same buffer, and at 4°C. With flow rates of 3–4 ml/min, the peroxidase rapidly separated from the $\text{Na}_2\text{S}_2\text{O}_4$ and took up oxygen from the solution phase. Spectrophotometry could be performed on the oxyferroperoxidase, which had a half-life of 35 min at 4°C.

Spectra were measured with either a Cary model 14 or a Unicam SP.1800 recording spectrophotometer with 1 cm-light-path cells in a thermostatically controlled cuvette holder.

Stopped-flow kinetic determinations were carried out with a Gibson–Durrum apparatus (Gibson & Milnes, 1964) with a 2 cm observation tube. Bandwidths were 1–2 nm and the dead time of the instrument was 3–4 ms.

Analogue computation was performed on a Solartron model 700 Hybrid series analogue computer and the simulated reaction was displayed in real time on a storage oscilloscope and photographed. The extinction changes were calculated from magnified tracings of developed film.

Results and Discussion

Oxyferroperoxidase: its spectra, properties and formation

Wittenberg *et al.* (1967) first demonstrated that a compound, oxyferroperoxidase, was formed when 1 mol. equiv. of ferroperoxidase reacted with 1 mol. equiv. of oxygen. The material produced has spectral characteristics recalling those of oxyhaemoglobin and oxymyoglobin. The absolute absorption spectra of ferri-, ferro- and oxyferro-peroxidase are shown in Fig. 1. That the material resembles other oxyhaem proteins spectrally is no proof of its identity. However, in a careful study Wittenberg *et al.* (1967) demonstrated the necessary stoichiometry and reported details of the second-order kinetics in its formation from ferroperoxidase and oxygen. These authors also suggested that compound III, the product of the reaction of ferriperoxidase with an excess of H_2O_2 might be oxyferroperoxidase.

It differs from other oxyhaem proteins in two ways. (1) The oxyferroperoxidase complex does not appear to dissociate, as established by the failure of high light intensity to photodissociate the complex and the

Fig. 1. Absolute absorption spectra from 500 to 650 nm of ferriperoxidase (a), ferroperoxidase (b) and oxyferroperoxidase (c)

For details see the text. ϵ_{mM} is in litre $\cdot$ mmol $^{-1}$ $\cdot$ cm $^{-1}$.

failure of CO or NO to trap any dissociated ferroperoxidase. (2) It decays rapidly to ferriperoxidase in a reaction which does not involve ferroperoxidase as an observable intermediate. The time-course of the decay of a freshly prepared oxyferroperoxidase solution to ferriperoxidase is shown in Fig. 2, and Fig. 3 shows the effect of temperature on this rate of conversion. Considerable difficulty would be encountered in studying this reaction if oxyperoxidase were generated independently for each experiment, because of the great instability of this complex. Kinetic experiments were therefore designed in which the material was generated *in situ* by rapidly mixing solutions of ferroperoxidase with various concentrations of oxygen-containing buffers. Since the ferroperoxidase might contain excess of $\text{Na}_2\text{S}_2\text{O}_4$, the real oxygen concentration after mixing has to be calculated from the amount of oxyferroperoxidase formed at low oxygen concentrations, and the concentration of ferroperoxidase can similarly be calculated from the amount of oxyferroperoxidase formed at high oxygen concentration. By proper selection of the monitoring wavelength it is then possible to determine (a) the amount of oxyferroperoxidase formed and (b) the amount of ferriperoxidase produced at the end of the experiment.

The spectral data used in this work are shown in Figs. 4(a), 4(b) and 4(c), which display the difference spectra ferro- minus ferri-peroxidase, oxyferro- minus ferro-peroxidase and ferri- minus oxyferroperoxidase respectively.

From these data in a series of experiments where 50 μM -ferroperoxidase was mixed with buffer solutions containing 13.5–270 μM -oxygen, stoichiometry was observed for the production of oxyferroperoxidase and ferriperoxidase from ferroperoxidase

and oxygen. Some of the traces obtained at a variety of monitoring wavelengths are shown in Fig. 5. At 532.5nm only the fast appearance of oxyferroperoxidase is seen, since this wavelength is isosbestic between ferro- and ferri-peroxidase. Similarly, at 548nm only the production of ferriperoxidase is seen as this wavelength is isosbestic between oxyferro- and ferro-peroxidase. At other wavelengths the kinetics are complex, as shown in the reaction profiles displayed in Fig. 5.

Fig. 2. Decay of oxyferroperoxidase to ferriperoxidase

The oxyferroperoxidase was generated by applying dithionite-reduced ferriperoxidase to a column of Sephadex G-25 as described in the Experimental section. The concentration of oxyferroperoxidase eluted was $90\mu\text{M}$ and spectral scans were performed at the following times: (1) immediately after preparation; (2) after 3min; (3) 7min; (4) 10min; (5) 22min; (6) 30min; (7) 50min; (8) 145min. The temperature was 12°C , and light-path 1cm. ϵ_{mM} is in litre $\cdot$ mmol $^{-1} \cdot \text{cm}^{-1}$.

The titration of ferropoxidase with oxygen to produce oxyferroperoxidase, as derived from ΔE measurements of the fast phase of the kinetics, is shown in Fig. 6, where the extrapolated end point of this titration agrees with the observed concentration of ferropoxidase originally present.

The rate constant for the formation of this complex taken from experiments at high oxygen concentration, where pseudo-first-order conditions apply, is $6.0 \times 10^4 \text{M}^{-1} \cdot \text{s}^{-1}$, which agrees well with the value given by Wittenberg *et al.* (1967). Alternatively, from the ΔE values obtained in the fast reaction, the amount of ferropoxidase and of oxygen can be determined at different times. A plot of $\log([\text{ferropoxidase}]/[\text{oxygen}])$ versus time gives a straight line whose slope

Fig. 3. Temperature-dependence of the decay of oxyferroperoxidase

A solution of oxyferroperoxidase prepared at 4°C as described in the Experimental section was transferred to a 1cm cuvette and the decay of oxyferroperoxidase to ferriperoxidase was followed with time at 578nm at (a) 9°C ; (b) 12°C ; (c) 16°C ; (d) 22°C .

Fig. 4. Difference spectra between (a) ferropoxidase and ferriperoxidase, (b) oxyferroperoxidase and ferropoxidase and (c) ferriperoxidase and oxyferroperoxidase

For details see the text. $\Delta\epsilon_{\text{mM}}$ is in litre $\cdot$ mmol $^{-1} \cdot \text{cm}^{-1}$.

Fig. 5. Tracings from stopped-flow experiments showing the time-course of reaction of $50 \mu\text{M}$ -ferroperoxidase with buffer solution containing oxygen ($54 \mu\text{M}$) at a series of observation wavelengths

(a) $\lambda = 532.5 \text{ nm}$; only a single fast process is seen corresponding to the formation of oxyferroperoxidase. Two traces on different time-scales are shown. (b) $\lambda = 548 \text{ nm}$; only a single slow process is seen, corresponding to the formation of ferriperoxidase from oxyferro- and ferroperoxidase. At other wavelengths, (c) $\lambda = 480 \text{ nm}$ and (d) $\lambda = 560 \text{ nm}$, composite profiles are recorded.

Fig. 6. Titration of ferroperoxidase with oxygen

The data were derived from the magnitude of the fast ΔE change observed in rapid-mixing experiments where $50 \mu\text{M}$ -ferroperoxidase was mixed with buffer solutions containing various oxygen concentrations. The arrow shows the titration point.

Fig. 7. Apparent pseudo-first-order plot of the formation of oxyferroperoxidase

From the magnitude of the fast ΔE change and the difference spectra shown in Fig. 4, the ratio of [ferroperoxidase]/[oxygen] can be calculated as a function of time. The second-order rate constant for this process is $5.6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$.

(multiplied by 2.303) gives the second-order rate constant. This is shown in Fig. 7, from which it can be concluded that $k = 5.6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Finally, the ΔE values of the fast initial reaction obtained by plotting the ΔE values obtained from the progress curve on semi-logarithmic paper versus time and subtracting where necessary the slow component of ΔE are shown in Fig. 8, superimposed on the difference spectra of oxyferro- minus ferro-peroxidase. The considerable agreement shows that there is confirmation for the belief that oxyferroperoxidase is the species formed in the initial reaction.

Fig. 8. Kinetic difference spectrum (○) of the fast ΔE change from a series of experiments normalized to the amount of oxyferroperoxidase produced, superimposed on the static difference spectrum of oxyferroperoxidase minus ferroperoxidase (—)

For details see the text.

Decay of oxyferroperoxidase

The oxyferroperoxidase formed by rapid mixing of ferroperoxidase with oxygen-containing solutions decays at a rate very much faster than that seen for oxyperoxidase alone. On the same arguments as those outlined above, the spectral dependency of the slow ΔE change should give information on the species present. If the above formulation of a subsequent reaction scheme is correct then the difference

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Fig. 9. Kinetic difference spectrum (○) of the slow ΔE change from a series of experiments which have been normalized on the basis of the amounts of ferro- and oxyferroperoxidase remaining after the fast phase of ΔE change is complete

These data are superimposed on the difference spectrum of (1 oxyferro- + 3 ferro-) minus 4 ferriperoxidase (—). $\Delta\epsilon_{mM}$ is in litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$.

spectrum recorded for this slow component should approximate to that for the difference spectra of (1 oxyferro- + 3 ferro-) minus 4 ferriperoxidase. Such a plot is shown in Fig. 9, with the values obtained from a number of experiments superimposed where the total ΔE of the slow reaction has been normalized on the basis of the amounts of ferro- and oxyferroperoxidase remaining after the fast phase of the reaction is completed.

Fig. 9 shows that reasonable agreement exists between the postulated scheme and the experimental results. Static spectrophotometry reveals that all the peroxidase used in an experiment was converted into the ferri form.

Mechanism of decay

Under conditions where ferroperoxidase is in excess

over oxygen, the sequence of events leading to ferriperoxidase and water may be described with a minimum of two steps:

Reaction 2 involves a four-body four-electron reaction, and a minimum requirement for a plausible scheme may be:

The kinetics observed in the millisecond range after rapid mixing appear free from spectrally distinct intermediates. It is thus concluded that the initial reaction whereby an electron is added to $\text{Fe}^{2+} \cdot \text{O}_2$ is rate-limiting (k_{slow}) and all other reactions proceed with very much higher rates (k_{fast}).

The kinetic difference spectra shown in Fig. 9 show that the stoichiometry of the overall scheme is obeyed. What is now required is the rate of the process. The second-order rate constant for the slow ΔE change might be calculated from the concentrations of oxyferriperoxidase and ferriperoxidase at different times, and a plot of $\log([\text{oxyferriperoxidase}]/[\text{ferriperoxidase}])$ versus time should produce a straight line whose slope (multiplied by 2.303) gives a value for the rate constant k' . This is related to the rate-limiting step in the scheme outlined above: $k' = 3 \times k_{\text{slow}}$. Attempts to derive this quantity were frustrated by the difficulty of disentangling reliably the contribution of the fast ΔE change corresponding to oxyferriperoxidase production from the slower following reaction in which it decayed to ferriperoxidase.

A further uncertainty is that the dithionite concentration was probably changing with time during the experiment.

Analogue computation

One method of testing the applicability of the overall scheme suggested above is to use analogue computation to simulate the whole reaction sequence and to compare (a) the adequacy of fit of experimental data with computed values and (b) to vary the experimental parameters to test the exclusivity of the scheme.

The equations used were:

$$-\frac{d\text{O}_2}{dt} = k_1[\text{O}_2][\text{Fe}^{2+}]$$

$$-\frac{d\text{Fe}^{2+}}{dt} = k_1[\text{O}_2][\text{Fe}^{2+}] + 3k_2[\text{Fe}^{2+} \cdot \text{O}_2][\text{Fe}^{2+}]$$

$$\frac{d\text{Fe}^{2+} \cdot \text{O}_2}{dt} = k_1[\text{O}_2][\text{Fe}^{2+}] - k_2[\text{Fe}^{2+} \cdot \text{O}_2][\text{Fe}^{2+}]$$

$$4 \frac{d\text{Fe}^{3+}}{dt} = k_2[\text{Fe}^{2+} \cdot \text{O}_2][\text{Fe}^{2+}]$$

The output displayed was the sum, $\varepsilon'[\text{Fe}^{2+}] + \varepsilon''[\text{Fe}^{2+} \cdot \text{O}_2] + \varepsilon'''[\text{Fe}^{3+}]$, where ε' is the molar absorptivity of ferriperoxidase; ε'' , that of oxyferriperoxidase and ε''' , that of ferriperoxidase.

The conditions for the best fit of experimental data are shown in Table 1. Only two parameters were fixed, those of ferriperoxidase concentration and the rate constant for oxyferriperoxidase production. Figs. 10 and 11 show that the agreement between experiment and prediction is good, both in the expected progress curves at various wavelengths and in the magnitude of the extinction changes with time at one fixed wavelength.

Moreover, it could be shown that the progress curves were exceedingly sensitive to the choice of molar absorptivities, and departures of 5% from the values shown in Table 1 led to unacceptable progress curves. The value of k_2 derived from these computations was best fitted to the experimental data at a value of $1.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (i.e. $3k_2 = 4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$).

General discussion

Oxyferriperoxidase is comparatively stable when there is no ferriperoxidase present and has a half-time

Table 1. Analogue computation: parameters used to obtain best fit of data to experiment

Values asterisked* were varied in the computation and the values given were those for best fit with experimental data. Ferriperoxidase: initial concentration $25 \mu\text{M}$. Oxygen*: initial concentration = 10 and $20 \mu\text{M}$. k_1 , rate constant for formation of oxyferriperoxidase, $= 6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. $3k_2^*$, rate constant for initial step in oxyferriperoxidase decay, $= 4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. Static data from spectra shown in Fig. 1 are compared with data in parentheses used for best fit.

λ (nm)	Extinction coefficients (ε_{mM} in litre $\cdot$ mmol $^{-1} \cdot \text{cm}^{-1}$) used in computation		
	Fe^{2+}	$\text{Fe}^{2+} \cdot \text{O}_2$	Fe^{3+}
530	6.8 (5.8)*	8.9 (8.9)	9.2 (8.0)*
560	11.8 (11.8)	8.3 (9.4)*	5.0 (5.0)*
580	7.5 (7.4)*	9.6 (9.6)	3.7 (3.7)*
600	3.7 (3.7)	2.2 (2.2)*	2.5 (2.5)*

Fig. 10. Comparison between analogue computer simulation of the reaction and the experimental data for progress curves at various wavelengths

The progress curves of the reactions are shown at four different wavelengths ($\circ$, 560 nm; $\bullet$, 580 nm; Δ , 600 nm; $\square$, 530 nm) derived from experimental data superimposed on the simulated reaction (—). The parameters used to establish this fit are given in Table 1.

of decay to ferriperoxidase of many minutes. However, when ferriperoxidase is present, oxyferriperoxidase acts as an oxidase since it accelerates the formation of ferriperoxidase.

The previous discussion has shown that the observed kinetics fit closely to a scheme:

and it is noteworthy that no intermediates were observable in the millisecond time-scale. This indicates that the rate-limiting step must be the addition of an electron to oxyferriperoxidase to yield a short-lived highly reactive species.

It may be of interest to speculate as to the nature of the intermediates X and Y in the simple scheme outlined above. Horseradish peroxidase can exist in five different iron-oxidation states. With ferro- and ferriperoxidase the oxidizing equivalents reside completely in the haem group. With compounds II, I and III, which are respectively at states +4, +5 and +6, the oxidizing equivalents are delocalized from the metal and are shared with the ligand. Thus there are a series of one-electron changes in state of the peroxidase. The formal valency required for intermediates X and Y in the scheme above are +5 and +4 respectively, though

Fig. 11. Comparison of the analogue-computer simulation of the reaction and the experimental data for the magnitude of extinction changes at one wavelength

The progress curves of the reaction are shown at one wavelength (560 nm) as followed at two oxygen concentrations ($\circ$, 10 μM ; $\bullet$, 20 μM). Experimental points ($\circ$, $\bullet$) are superimposed on the simulated reaction profile (—).

there is no direct evidence to identify these intermediates with compounds I and II.

There is a further significance in the present results. It has been known for a long time that autoxidations of haem proteins occur faster in mixtures of Fe^{2+} and $\text{Fe}^{2+} \cdot \text{O}_2$ proteins than in single species. Thus the original observation by Neill & Hastings (1925), that the autoxidation of ferrohaemoglobin is determined by its degree of oxygen saturation, was extended by the work of Brooks (1932, 1935, 1948), who showed the reaction velocity was directly proportional to the ferrohaemoglobin concentration. Also in the case of the autoxidation of myoglobin the rate is first-order with respect to ferrohaemoglobin (George & Stratmann, 1952a,b). Though no generally satisfactory explanation has yet been found for the autoxidation of haem proteins, the general similarities to the work described in the present paper prompt belief that the scheme outlined above may be correct.

In cytochrome oxidase, Greenwood & Gibson (1967) have suggested that a molecule of oxygen enters the functional complex of two haem groups and two copper atoms. There sequentially, electrons are transferred, first from haem a_3 then from two copper atoms and lastly from haem a . The role of the so-called oxygenated oxidase in the reaction is still open

to speculation, but it appears that this compound may be formed under conditions similar to that for oxyferroperoxidase and has similar decay properties (Lemberg, 1969; Greenwood *et al.*, 1974). Thus the fully oxygenated complex is fairly stable and decays slowly to ferric oxidase. On the other hand, 'oxygenated' oxidase disappears very rapidly in the presence of cytochrome *c*.

As shown for the oxidase systems, in all cases where oxidation is fast, there is a multi-electron donation to oxygen (Antonini *et al.*, 1970). This situation is simulated in the present case by the intermolecular electron transfer between oxyferroperoxidase and ferropoxidase.

The place oxyferroperoxidase occupies in peroxidase reactions is still obscure. Its position has improved from that of being a denaturation product caused by excess of H_2O_2 , and the identity of compound III with oxyferroperoxidase seems likely. In contrast with the peroxidatic behaviour involving the ferric species and the peroxide adducts, peroxidases also catalyse aerobic oxidations of, typically, dihydroxyfumarate, without cofactors, where oxyferroperoxidase has been observed as an intermediate. This oxidase action is inhibited by catalase, cyanide, fluoride and azide, but also by CO. It thus seems that

there may be a real role for the oxyferroperoxidase species, at present little explained.

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