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The Mechanism of Haem Degradation *in vitro*

KINETIC EVIDENCE FOR THE FORMATION OF A HAEM–O₂ COMPLEX

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The rate of haem degradation in aqueous pyridine shows a saturation dependence on O₂ concentration. CO competitively inhibits the reaction. This is evidence for formation of an iron–O₂ complex and is consistent with an intramolecular pathway for haem degradation.

Haem degradation may be readily studied in several types of system, ranging from the intact animal to chemical model systems (Jackson, 1974; O'Carra, 1975; Schmid & McDonagh, 1975). The model systems usually involve treatment of haem with ascorbate and O₂ in aqueous pyridine, a reaction known as coupled oxidation (Jackson, 1974). In these model reactions it seems likely that one function of the ascorbate is the initial reduction of Fe(III) to Fe(II). Studies of the involvement of O₂ in the reaction in intact animals (Brown & King, 1978), in the cell-free haem oxygenase system (King & Brown, 1978) and in coupled oxidation of haemoproteins (Brown & King, 1976a) and octaethylhaemin (Chaney & Brown, 1978) have suggested that there is a single mechanism for bile-pigment production from haem, i.e. that the non-biological systems represent good models for the reaction *in vivo*. The precise way in which O₂ is involved in the reaction has not been determined. This is particularly relevant to the very first step, in which activation of O₂ takes place with hydroxylation at a methene bridge to yield oxyhaem (iron oxophlorin; Jackson, 1974). The subsequent steps, involving conversion of oxyhaem to bile pigment must involve several other intermediates, but it seems likely that these steps are fast and that the rate of bile-pigment formation is effectively determined by the rate of the initial hydroxylation step (Jackson, 1974). In an attempt to account for the unexplained role of apoproteins in determining methene bridge selectivity during haem cleavage, Brown (1976) suggested that the hydroxylation step occurred by a wholly intramolecular reaction involving oxygen activation by the iron atom followed by intramolecular attack at the appropriate methene bridge. The formation of an iron–O₂ complex is a requirement of this scheme. Studies on the coupled oxidation of mixtures of haem with iron-free porphyrins resulted in degradation only of the haem (Brown & Grundy, 1977). This result was interpreted as support for the intramolecular degradation hypothesis. However, direct proof of such a process is hitherto not available.

To investigate the possibility of an intramolecular reaction further, in the present work we report kinetic studies of the dependence of the rate of haem-coupled oxidation on the concentration of O₂. Two general reaction schemes A and B may be distinguished:

Scheme A envisages reaction occurring as a direct oxidation of a methene bridge by O₂, not attached to the haem iron atom. In principle, the attacking O₂ could be bound to the iron atom of another haem molecule, but this seems unlikely on the basis of the results of Brown & Grundy (1977) and would be impossible in the haemoproteins. Scheme B involves the formation of a haem–O₂ complex and is compatible with the intramolecular pathway for degradation. Several variants of these schemes are possible and it is clear that the steps may be composite, but the essential difference between them is that Scheme B proceeds via a kinetically distinct haem–O₂ complex, whereas no such complex is formed in Scheme A. These schemes predict different kinetic forms for the dependence of the reaction rate on O₂ concentration. Scheme A leads to a single expression for the reaction rate, v :

$$v = k_{+1}[\text{haem}][\text{O}_2]$$

Provided that the initial rate is measured (i.e. [haem] is constant) and that [O₂] ≫ [haem], Scheme B is exactly analogous to the classical Michaelis–Menten model of enzyme catalysis and leads to the rate expression:

$$v_0 = \frac{k_{+2}[\text{haem}][\text{O}_2]}{K + [\text{O}_2]} \quad (1)$$

where v_0 is the initial degradation rate and:

$$K = \frac{k_{+2} + k_{-1}}{k_{+1}} \quad (2)$$

The two reaction schemes may therefore be distinguished experimentally, since Scheme B predicts a saturation dependence on $[O_2]$, whereas Scheme A predicts a strictly linear dependence on $[O_2]$.

Experimental

Protohaemin, ascorbic acid and pyridine were obtained as previously described (Brown & King, 1976b). Ferrihaem solutions were prepared by appropriate dilutions of stock 0.48 mM solutions in pure pyridine and measurements were made within 8 h of dilution. Gas mixtures were obtained in two ways. For binary mixtures of N_2 and O_2 , pressurized cylinders containing mixtures of known composition were supplied by the Anaesthetics Department, University of Leeds. Some additional N_2/O_2 mixtures and all mixtures containing CO were prepared at atmospheric pressure by use of an inverted 1-litre measuring cylinder, initially filled with water. Results from O_2/N_2 mixtures obtained in both ways were consistent within the limits of experimental error.

Reactions were carried out at 25°C in specially constructed spectrophotometer cuvettes of 1 cm path length. Before initiation of reaction, 3.9 ml of ferrihaem solution in 25% (v/v) pyridine/water was placed in the reaction cuvette, which was sealed with a Suba-Seal (Gallenkamp and Co, London EC2P 2ER, U.K.) that remained gas-tight after insertion and removal of syringe needles. A steady stream of the appropriate gas mixture was bubbled through the solution (at 25°C) for 10 min via two fine syringe needles inserted through the rubber seal. Trial experiments showed that complete equilibration occurred within this time. The reaction was initiated by injection of 0.1 ml of 5% (w/v) ascorbic acid (freshly prepared) and the cuvette quickly placed in the spectrophotometer (Pye-Unicam SP. 1800). All reaction rates were measured at this constant ascorbate concentration. The initial reaction rate was determined by following the rate of absorbance increase at 650 nm. Chart-recorder traces were linear for the first 0.5–1 min of reaction. Since the molar absorption coefficient of the immediate haem-breakdown product (probably a biliverdin-iron complex) at 650 nm is not accurately known, v_0 values were expressed in terms of change in absorbance units per min.

Results and Discussion

To express O_2 concentrations in terms of molarity, the value of the solubility of O_2 in 25% (v/v) pyridine/water at 25°C is required. This was derived from literature data (Linke, 1965) as 1.22 mM under 101 kPa (1 atm) of pure O_2 . Similarly, the solubility of CO in 25% (v/v) pyridine/water at 25°C was derived from solubility tables (Linke, 1958) as 0.97 mM under 101 kPa of pure CO. It was assumed that for gas

mixtures, the solubility of a component gas was proportional to its partial pressure. Corrections for atmospheric pressure variation were insignificant.

Fig. 1(a) shows the variation in v_0 with O_2 concentration for three different haem concentrations. It is apparent that a saturation effect occurs as O_2 concentration increases, although complete saturation (corresponding to a maximum value of v_0) has not been reached at any haem concentration. This kinetic form is therefore evidence in favour of Scheme B and against Scheme A. {Although $[O_2]$ is always in excess of $[haem]$, the condition $[O_2] \gg [haem]$ does not hold for a few of the points in Fig. 1(a) at low $[haem]$.} From eqn. (1)

$$\frac{1}{v_0} = \frac{K}{k_{+2}[haem]} \frac{1}{[O_2]} + \frac{1}{k_{+2}[haem]} \quad (3)$$

With this relationship a plot of $1/v_0$ against $1/[O_2]$ should be linear, analogous to the double-reciprocal

Fig. 1. Dependence of rate of haem-coupled oxidation on O_2 concentration

The initial rate v_0 is expressed in terms of change in absorbance at 650 nm per min. (a) Plots of v_0 against $[O_2]$. Haem concentrations were: ●, 60 μM ; △, 30 μM ; ○, 15 μM . (b) Double-reciprocal plots of the data in (a) above. Haem concentrations and symbols as above. The broken lines refer to regions where the condition $[O_2] \gg [haem]$ does not strictly hold (see the text).

plot of enzyme kinetics, except at low [haem] where the condition $[O_2] \gg [haem]$ does not hold. The data of Fig. 1(a) are plotted in this way in Fig. 1(b). Within experimental error they are consistent with eqn. (3), except for small deviations at low $[O_2]$, presumably owing to the relatively small excess of $[O_2]$ over [haem]. Also, the lines for different haem concentrations pass through a common point on the $1/[O_2]$ axis. This is expected, since the intercept on the $1/[O_2]$ axis is equal to K , which should be independent of [haem]. The value of K determined from these data is 0.31 mM. The results are therefore consistent with the formation of a haem- O_2 complex as an integral part of the pathway of haem degradation.

To study the site of O_2 binding further, kinetic measurements similar to those shown in Fig. 1 were carried out in presence of small concentrations of CO, which competes very effectively with O_2 for binding at the iron atom of haemoproteins. Fig. 2(a) shows

Fig. 2. Inhibition of rate of haem-coupled oxidation by CO. The initial rate, v_0 , is expressed in terms of change in absorbance at 650 nm per min. (a) Plots of v_0 against $[O_2]$ at a constant haem concentration of 30 μM . Concentrations of CO were: $\bullet$, 0.048 mM; Δ , 0.020 mM; $\circ$, zero. (b) Double-reciprocal plots of the data in (a). Concentrations and symbols as above.

two sets of results with CO partial pressures of 5 and 2 kPa (0.05 and 0.02 atm) respectively. Again the saturation effect is clearly observed, but in this case substantial inhibition by CO is apparent. If the inhibitory action of CO is due to a competition with O_2 for binding at the haem iron atom, then, except at the very lowest $[O_2]$ (see above), the form of the double-reciprocal plots should be identical with those for the competitive inhibition of enzyme kinetics, i.e. the lines should intersect on the $1/v_0$ axis. Fig. 2(b) shows double-reciprocal plots of the data in Fig. 2(a). Within experimental error, these lines do indeed intersect on the $1/v_0$ axis, thus confirming the competition between CO and O_2 .

K is not a true dissociation equilibrium constant for the haem- O_2 complex, since it embodies both k_{-1} and k_{+2} . However, reference to eqn. (2) shows that if k_{+2} is small compared with k_{-1} , corresponding to a rapid pre-equilibrium for O_2 binding, then the reciprocal of K is given by k_{+1}/k_{-1} , which equals the formation constant K_f for the haem- O_2 complex. In this case $K_f = 3.23 \times 10^3 M^{-1}$. In all cases where k_{+2} is not small compared with k_{-1} , eqn. (2) shows that k_{+1}/k_{-1} must be higher than this value. Thus the minimum value of K_f from this kinetic analysis is $3.23 \times 10^3 M^{-1}$. Unfortunately direct measurement of K_f under these conditions is not possible because of rapid oxidation of Fe(II) to Fe(III). The corresponding value of K_f for formation of oxymyoglobin is in the range 8×10^5 – $12 \times 10^5 M^{-1}$, depending on the species (Antonini & Brunori, 1971). This may mean that the affinity of O_2 for haem is very much higher for myoglobin than for protein-free haem or alternatively that k_{+2} is very much greater than k_{-1} in Scheme B and consequently $K_f \gg 3.23 \times 10^3 M^{-1}$.

The inhibition by CO may be represented by the scheme:

where K' is an inhibitor-binding constant given by $K' = [haem-CO]/[haem][CO]$. It is readily shown (e.g. Segel, 1976) that the equation for double-reciprocal plot, taking into account this competitive inhibition, is:

$$\frac{1}{v_0} = \left(\frac{K(1 + K'[CO])}{k_{+2}[haem]} \cdot \frac{1}{[O_2]} \right) + \frac{1}{k_{+2}[haem]} \quad (5)$$

By comparison of the intercepts on the $1/[O_2]$ axis in Fig. 2(b) with eqn. (5) the value of K' was found to be $5.9 \times 10^4 M^{-1}$. Assuming the minimum value of K_f calculated above, this suggests that CO has a maximum of about 18 times the affinity for haem as O_2 in these experiments. This is reasonable in view of the corresponding ratio for myoglobin of 40 (Antonini & Brunori, 1971).

On the basis of these results, it seems likely that haem degradation occurs via the formation of a

haem-O₂ complex in which the O₂ molecule is bound to the iron atom. An essential requirement of the intramolecular mechanism (Scheme B) has therefore been demonstrated. It is conceivable that the observed kinetic form would also be predicted by a mechanism in which an iron-bound activated oxygen moiety dissociated from its haem group to attack the methene bridge of another haem group. However, such a mechanism seems unlikely in view of the observed lack of degradation of iron-free porphyrins in the presence of haem (Brown & Grundy, 1977).

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