The Participation of Both $O_2^{\cdot -}$ and HO_2^- Species in the Haemin-catalysed *para*-Hydroxylation of Aniline

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Summary A haemin-containing model system for biological substrate hydroxylation has been studied and direct evidence for participation of both superoxide and hydroperoxide anions in the catalytic process is presented.

THE mono-oxygenase system of liver microsomal membranes is, by virtue of its ability to hydroxylate a variety of substances such as drugs, anaesthetics, and potential carcinogens, an important component of the detoxification–activation system of the liver. The *para*-hydroxyla-

tion of aniline provides a simple probe for the mechanistic investigation of such biological systems, and much work has been carried out on the *para*-hydroxylation of aniline by cytochrome P-450 (and other haemoproteins) as well as by model systems, particular interest centring on the nature of the active oxygen species participating in the hydroxylation.[1-5] A drawback to the use of models is that even in the best system studied, the thiol-containing peptide–haemin system, the yield of *p*-aminophenol is poor (*ca.* 0·5%) compared to the cytochrome P-450-mediated process.[5]

Haemin-catalysed hydroxylation would appear to be a simple and relevant model for haemoprotein catalysed hydroxylations, but does not appear to have been studied in any detail. The lack of such an investigation is probably due to the reportedly low hydroxylating ability of haemin at neutral pH.[6] We report in this communication the characterisation of a haemin-containing system which is a model for the cytochrome P-450-catalysed hydroxylation of aniline with respect to both the yield of *p*-aminophenol, and the apparent Michaelis constant (K_m) for aniline as substrate. Direct evidence is presented for participation of both $O_2^{\bullet-}$ and HO_2^- anions in the reaction pathway.

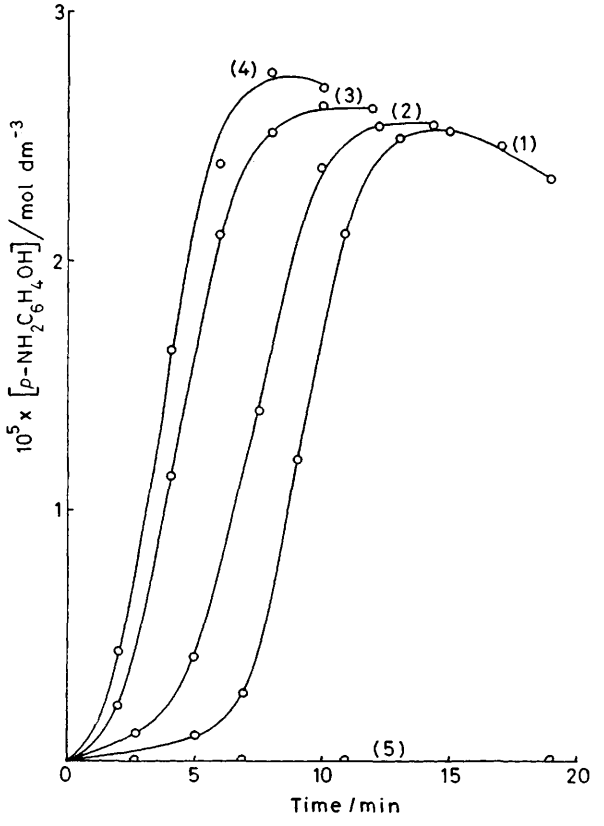

FIGURE. The hydroxylation of aniline to *p*-aminophenol at pH 11·45, 30·0 °C. (1): [haemin] = 5 × 10^{-6} mol dm^{-3}, [NADH] = 0·001 mol dm^{-3}, [aniline] = 0·050 mol dm^{-3}; (2): (1) + H_2O_2 (2·8 × 10^{-6} mol dm^{-3}); (3): (1) + H_2O_2 (5·5 × 10^{-6} mol dm^{-3}); (4): (1) + H_2O_2 (11·0 × 10^{-6} mol dm^{-3}); (5): as in (4) but no haemin added.

The hydroxylation system consisted of haemin (5 × 10^{-6} mol dm^{-3}), aniline (0—0·10 mol dm^{-3}), NADH or NADPH (0·001 mol dm^{-3}), and buffer (pH 7—12·8); the total reaction volume was 16·0 cm^3. Reaction was initiated by addition of stock haemin solution (25 μl in Me_2SO) to the stirred solution of aniline and NADH thermostatted at 30·0 °C. Aliquots of the reaction mixture (1 cm^3) were removed at fixed time intervals, and after quenching and removal of the haemin (*ca.* 99%) by precipitation as methaemalbumin, the amount of *p*-aminophenol formed was determined by the indophenol reaction.[4]

Typical hydroxylation curves obtained are shown in the Figure. Failure to remove haemin prior to the colour reaction stage results in additional formation of *p*-aminophenol occurring at the alkaline pH of the colour reaction (pH *ca.* 12·5); this could have given rise to previous conclusions concerning the apparent catalytic activity, with respect to *para*-hydroxylation of aniline, of haemin at neutral pH.[7]

Production of *p*-aminophenol ceases when the NADH is depleted, and further reaction can be initiated by injection of additional NADH at the maxima of the curves shown. Reduction of the induction phase by addition of H_2O_2 (Figure) strongly suggests that H_2O_2 is formed prior to, and is required for, catalysis of *para*-hydroxylation. This conclusion is confirmed on studying the pH variation of the maximum rate of *p*-aminophenol formation, a pK of 11·7 ± 0·1 (virtually identical to the pK of H_2O_2) being obtained for the process. This observation indicates direct involvement of the hydroperoxide anion in the catalytic process. No catalysis of *para*-hydroxylation is observed below pH 11.

Both O_2 and a reductant (NADH or NADPH) are required for hydroxylation; these requirements are similar to those of a mono-oxygenase system. Data in the Figure also indicate that haemin is required for catalysis subsequent to the formation of H_2O_2. No catalysis occurs in the presence of added H_2O_2 but the absence of haemin.

Addition of superoxide dismutase (final concentration 10^{-7} mol dm^{-3}) to the reaction mixture inhibits (>99%) the formation of *p*-aminophenol, and although normal hydroxylation commences after 25 min (presumably owing to alkaline denaturation of the dismutase), addition of more enzyme further inhibits the reaction. This observation proves the participation of superoxide anion ($O_2^{\bullet-}$) in the catalytic process.

The reaction follows typical binding kinetics with respect to aniline concentration, the apparent Michaelis constant, 2 (±0·2) × 10^{-3} mol dm^{-3}, comparing well with values of 0·1—6 × 10^{-3} mol dm^{-3} reported for aniline hydroxylation by liver microsomes from a variety of sources.[8] The yield of *p*-aminophenol at an aniline concentration of 1 × 10^{-3} mol dm^{-3} was 1·5% (minimum) compared to 0·47% for thiol-containing peptide–haemin complexes, and 5·5% for mouse liver microsomes.[5]

We have shown that haemin, the prosthetic group of cytochrome P-450, catalyses the *para*-hydroxylation of aniline at pH >11 with an efficiency approaching that of the enzyme. Both $O_2^{\bullet-}$ and HO_2^- anions have been shown to take part in the reaction, the latter being the species participating in the rate-determining step. The fact that haemin-catalysed hydroxylation only occurs at pH >11 suggests that the function of the protein portion of cytochrome P-450 in biological hydroxylation may be to

provide, in the micro-environment of the active site, a basicity mimicking a free solution pH >11. This would allow formation and stabilisation of HO_2^- by the haem at physiological pH.

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