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Mechanism of activation of H_2O_2 by peroxidases: kinetic studies on a model system

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Kinetic studies on the peroxidase activity of microperoxidase-8 at pH 5.5–8.5 show that the rate is increased by raising the pH or by the presence of guanidinium ion. Comparison with published data on the peroxidases provides evidence that the enzyme activates H_2O_2 through the cooperative binding of $H^+ + HO_2^-$ and suggests a role for the invariant distal Arg.

Peroxidase *Proton-coupled reaction* *Arginine* *Microperoxidase-8* *Hydrogen peroxide* *Guanidinium*

1. INTRODUCTION

Most peroxidases [1] and apparently all myoglobins (Mbs) [2] are monomeric haemoproteins with the same haem and the same proximal (His) ligand, yet H_2O_2 reacts far more rapidly with Fe^{III} in the peroxidases [3]; they provide the classic example amongst metallo-proteins of the ability of the protein to control function. The recent X-ray structural determination of yeast cytochrome *c* peroxidase identified the distal residues as His, Arg (both invariant in all peroxidases studied) and Trp (variable) [4,5] in contrast to, for example, His and Val in vertebrate Hbs and Mbs [2]; attention is therefore focussed on the role of the guanidinium side-chain of Arg.

To establish the role of the protein one must compare the properties of the co-factor with and without the protein. We have therefore studied the peroxidase activity of the haem-octapeptide (H8PT) or microperoxidase-8 (MP-8), using the assay based on Gc [6]. MP-8 is derived from cytochrome *c* and retains residues 14–21 with His 18 coordinated to the Fe [7]; it also retains the two thioether links to the haem present in the parent cytochrome, but studies with reconstituted peroxidases show that varying the side-chain at positions 2 and 4 has little effect on activity [8]. We summarise here our results on (i) the pH dependence of k_1 (the Gc-independent rate of reaction of H_2O_2 with Fe^{III} [6]) at pH < 9, where MP-8 is present as the aquo complex (the pK for formation of the hydroxo complex has been reported as 9.9 [7]), and (ii) the additional effect of $GuaH^+$. For earlier work on the peroxidase activity of MP-8 see [9,10].

2. MATERIALS AND METHODS

MP-8 (Sigma) was purified by a method based on that of [10] and concentrations determined by the pyridine haemochromogen method [11]. Gc (Riedel-de-Haën) was purified by distillation and stored under N_2 in the dark. $GuaH^+Cl^-$ (Sigma) and H_2O_2 (Saarchem) were used without further purification. The concentration of the latter was determined spectrophotometrically using $\epsilon_{240} = 39.4\ M^{-1} \cdot cm^{-1}$ [12]. Phosphate buffers were prepared according to [13]. UV-visible spectra

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Abbreviations: Gc, guiacol; $GuaH^+$, guanidinium ion; MP-8, microperoxidase-8

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were recorded with a Cary 2300 spectrophotometer in 1 cm cells at 25 ± 0.2°C.

3. RESULTS

If solutions containing ~8×10^{-7} M MP-8 and 3×10^{-3} M Gc at pH 6 or 7 are allowed to stand for ~2 h before adding H_2O_2 (1.4×10^{-4} M), then the plot of A_{470} vs time is linear before eventually slowing down (due to depletion of H_2O_2). If, however, the reaction is initiated by injecting a small volume of stock (~4×10^{-5} M) MP-8 into the solution of H_2O_2 and Gc, then the linear portion is preceded by a period of increasing rate, which we ascribe to the relatively slow formation of monomeric MP-8 from less active aggregated forms present in the more concentrated stock solution. This induction period was observed at pH ≤ 7, both in the presence and absence of $GuaH^+$ (see below), its duration increasing as the pH was lowered. For convenience subsequent reactions were initiated by injecting a stock solution of MP-8 and the catalytic activity determined from the linear portion of the kinetic trace. Varying the Gc concentration showed that the rate was virtually constant over a wide range (~2×10^{-4} to 6×10^{-3} M Gc at pH 6, 7 and 8) and linearly dependent on the concentration of both MP-8 and H_2O_2, i.e., the observed rate corresponds to k_1.

Fig.1 shows the effects of increasing concentrations of phosphate, KCl, $EtNH_3^+Cl^-$ and $GuaH^+Cl^-$ on k_1 at pH 7. Other substances tested include imidazole, which inhibits the reaction due to formation of the 6-coordinate imidazole adduct (λ_{max} 404 nm), and urea and acetamide (no effects). The addition of 0.5 M GuaHCl or KCl caused the same slight fall in the Soret band, when studied with a low (4×10^{-8} M) concentration of MP-8 in a 10 cm cell, i.e., the effect of GuaHCl does not appear to involve coordination to Fe.

The pH dependence of log k_1 in the absence of GuaHCl is shown in fig.2a; if one assumes that the hydroxo complex is catalytically inert and formed with $pK \leq 9.4$, then the data of curve a can be corrected to give an excellent linear plot (curve b) with a slope of 1.0 and log $k_1 = 6.3 \times 10^2$ $M^{-1} \cdot s^{-1}$ at pH 6.0. The analogous data in the presence of 0.5 M GuaHCl are shown in fig.2c; in this case, however, the best correction (using $pK \leq 8.4$) only gave a plot (curve d) with a slope of 1.3.

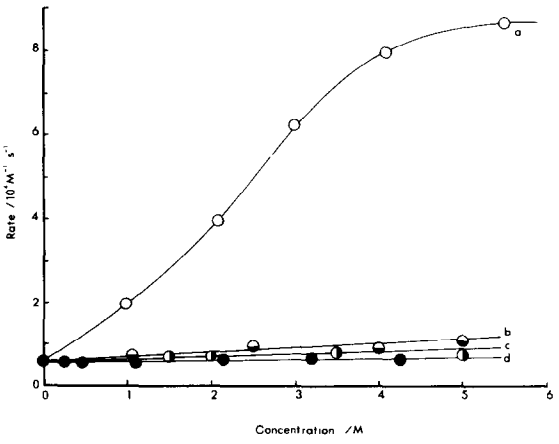

Fig.1. Effect of increasing concentration of (a) $GuaH^+Cl^-$, (b) $EtNH_3^+$, (c) KCl, and (d) K_2HPO_4 on the rate of reaction (k_1) of H_2O_2 with MP-8 at pH 7.

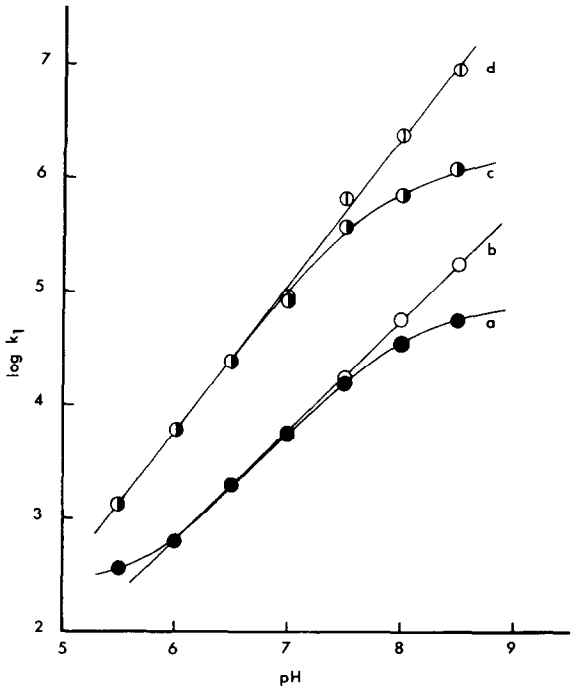

Fig.2. Effect of pH on log k_1 for the reaction of H_2O_2 with MP-8. (a) μ = 0.1 phosphate buffer; (b) after correcting (a) for pK_a ($FeOH_2$) ≤ 9.4; (c) (a) plus 0.5 M $GuaH^+Cl^-$; (d) after correcting (c) for pK_a ($FeOH_2$) ≤ 8.4.

4. DISCUSSION

Our results establish two points. (A) The pH dependence of fig.2a,b, together with the pK values for ionisation of H_2O_2 (11.7 [14]) and for formation of the hydroxo complex (9.4–9.9), indicate that the rate-determining step involves reaction of the aquo complex with HO_2^-. (B) Fig.1 shows that $GuaH^+$ (representing the side-chain of Arg) has an accelerating effect not shown by $EtNH_3^+$ (cf. Lys), while the pH dependence (fig.2c,d) shows that $GuaH^+$ must interact with HO_2^-; this can probably be ascribed to an ability to bind HO_2^- by two H-bonds (each from one NH of $GuaH^+$ to one O of HO_2^-), as observed in the crystalline adduct of urea with H_2O_2 [15]. The effect of uncoordinated imidazole could, unfortunately, not be tested.

Point A completes the evidence required to demonstrate that the Fe^{III} ion in MP-8 behaves 'normally' towards species with a single negative charge (e.g., reaction with HO_2^-, coordination of N_3^- [16], uptake of an electron [7]), while the Fe^{III} ion in the peroxidases shows the same abnormality (viz. simultaneous uptake of H^+ or equivalent loss of HO^-) with all 3 species [3]. The positive correlation between the ability of horseradish peroxidase to activate H_2O_2 and the need to bind H^+ with F^- [17] provides evidence for a common denominator in all these abnormalities. Extrapolation of curve b in fig.2 gives a value of log $k = 8.4$ for reaction of MP-8 with the fully formed HO_2^- (assuming pK 11.7 [14]), which is close to the value of log $k = 8.1$ reported [18] for the reaction of cytochrome c peroxidase with H_2O_2; this provides evidence for a common denominator in the reactions of MP-8 and peroxidase after allowance is made for the abnormal proton uptake by the peroxidases. The primary role of the protein is, therefore, to convert the simple reaction with HO_2^-, as observed with MP-8, into a proton-coupled reaction; this enables the enzyme to bind H_2O_2 as H^+ and the required HO_2^- in a pH-independent equilibrium [3]. We have provided protein-free models for the proton-coupled reduction of haemoproteins [19,20] and have suggested Arg as the site of proton uptake in the enzymes [19], but the recently demonstrated conformational flexibility of the Arg side-chain [21] obviously complicates attempts to identify the site of proton uptake; the flexible Arg may, for example, act as mediator for the overall transfer of H^+ from H_2O_2 to one of the suitably placed [21] and apparently essential [8] carboxylate side-chains of the haem. Point B also demonstrates a role for Arg unrelated to the mechanism of proton uptake and suggests a reason for the invariant distal Arg in the peroxidases; the formation of the analogue of $GuaH^+HO_2^-$ could increase the local concentration of HO_2^- required for coordination to Fe^{III} in the first step and/or facilitate the transfer of H^+ to the uncoordinated O atom (to give FeO^{3+} and H_2O) in the second step of the reaction.

The peroxidases provide the first family of metallo-enzymes where we can follow changes in catalytic activity at the 3 separate stages of co-factor alone, co-factor + accessories (viz. Arg/$GuaH^+$), and holoenzyme. Full experimental details and further discussion will be published elsewhere.

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