

# Mechanism of activation of $\text{H}_2\text{O}_2$ 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  $\text{H}_2\text{O}_2$  through the cooperative binding of  $\text{H}^+ + \text{HO}_2^-$  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  $\text{H}_2\text{O}_2$  reacts far more rapidly with  $\text{Fe}^{\text{III}}$  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  $\text{H}_2\text{O}_2$  with  $\text{Fe}^{\text{III}}$  [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  $\text{GuaH}^+$ . 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  $\text{N}_2$  in the dark.  $\text{GuaH}^+\text{Cl}^-$  (Sigma) and  $\text{H}_2\text{O}_2$  (Saarchem) were used without further purification. The concentration of the latter was determined spectrophotometrically using  $\epsilon_{240} = 39.4 \text{ M}^{-1} \cdot \text{cm}^{-1}$  [12]. Phosphate buffers were prepared according to [13]. UV-visible spectra

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**Abbreviations:** Gc, guaiacol;  $\text{GuaH}^+$ , guanidinium ion; MP-8, microperoxidase-8

were recorded with a Cary 2300 spectrophotometer in 1 cm cells at  $25 \pm 0.2^\circ\text{C}$ .

### 3. RESULTS

If solutions containing  $\sim 8 \times 10^{-7}$  M MP-8 and  $3 \times 10^{-3}$  M Gc at pH 6 or 7 are allowed to stand for  $\sim 2$  h before adding  $\text{H}_2\text{O}_2$  ( $1.4 \times 10^{-4}$  M), then the plot of  $A_{470}$  vs time is linear before eventually slowing down (due to depletion of  $\text{H}_2\text{O}_2$ ). If, however, the reaction is initiated by injecting a small volume of stock ( $\sim 4 \times 10^{-5}$  M) MP-8 into the solution of  $\text{H}_2\text{O}_2$  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  $\text{pH} \leq 7$ , both in the presence and absence of  $\text{GuaH}^+$  (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 ( $\sim 2 \times 10^{-4}$  to  $6 \times 10^{-3}$  M Gc at pH 6, 7 and 8) and linearly dependent on the concentration of both MP-8 and  $\text{H}_2\text{O}_2$ , i.e., the observed rate corresponds to  $k_1$ .

Fig.1 shows the effects of increasing concentra-

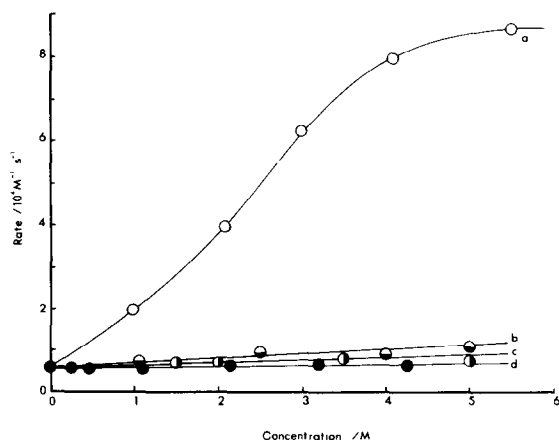


Fig.1. Effect of increasing concentration of (a)  $\text{GuaH}^+\text{Cl}^-$ , (b)  $\text{EtNH}_3^+$ , (c) KCl, and (d)  $\text{K}_2\text{HPO}_4$  on the rate of reaction ( $k_1$ ) of  $\text{H}_2\text{O}_2$  with MP-8 at pH 7.

tions of phosphate, KCl,  $\text{EtNH}_3^+\text{Cl}^-$  and  $\text{GuaH}^+\text{Cl}^-$  on  $k_1$  at pH 7. Other substances tested include imidazole, which inhibits the reaction due to formation of the 6-coordinate imidazole adduct ( $\lambda_{\text{max}}$  404 nm), and urea and acetamide (no effects). The addition of 0.5 M  $\text{GuaHCl}$  or KCl caused the same slight fall in the Soret band, when studied with a low ( $4 \times 10^{-8}$  M) concentration of MP-8 in a 10 cm cell, i.e., the effect of  $\text{GuaHCl}$  does not appear to involve coordination to Fe.

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

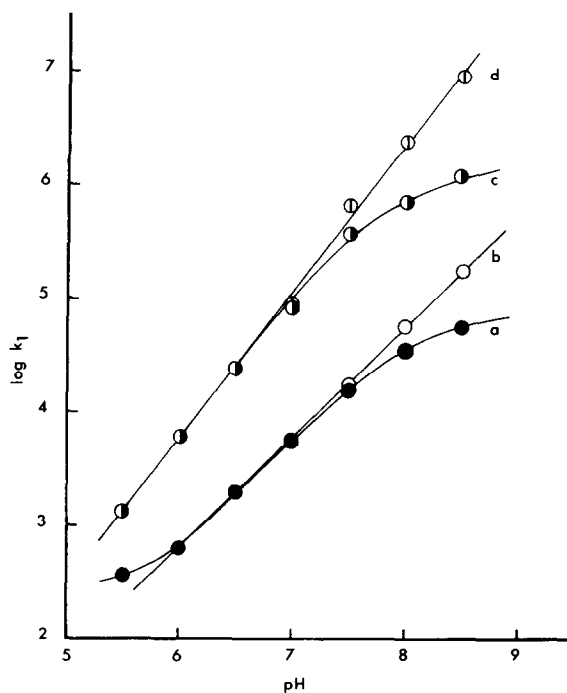


Fig.2. Effect of pH on  $\log k_1$  for the reaction of  $\text{H}_2\text{O}_2$  with MP-8. (a)  $\mu = 0.1$  phosphate buffer; (b) after correcting (a) for  $\text{pK}_a(\text{FeOH}_2) \leq 9.4$ ; (c) (a) plus 0.5 M  $\text{GuaH}^+\text{Cl}^-$ ; (d) after correcting (c) for  $\text{pK}_a(\text{FeOH}_2) \leq 8.4$ .

#### 4. DISCUSSION

Our results establish two points. (A) The pH dependence of fig.2a,b, together with the pK values for ionisation of  $\text{H}_2\text{O}_2$  (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  $\text{HO}_2^-$ . (B) Fig.1 shows that  $\text{GuaH}^+$  (representing the side-chain of Arg) has an accelerating effect not shown by  $\text{EtNH}_3^+$  (cf. Lys), while the pH dependence (fig.2c,d) shows that  $\text{GuaH}^+$  must interact with  $\text{HO}_2^-$ ; this can probably be ascribed to an ability to bind  $\text{HO}_2^-$  by two H-bonds (each from one NH of  $\text{GuaH}^+$  to one O of  $\text{HO}_2^-$ ), as observed in the crystalline adduct of urea with  $\text{H}_2\text{O}_2$  [15]. The effect of uncoordinated imidazole could, unfortunately, not be tested.

Point A completes the evidence required to demonstrate that the  $\text{Fe}^{\text{III}}$  ion in MP-8 behaves 'normally' towards species with a single negative charge (e.g., reaction with  $\text{HO}_2^-$ , coordination of  $\text{N}_3^-$  [16], uptake of an electron [7]), while the  $\text{Fe}^{\text{III}}$  ion in the peroxidases shows the same abnormality (viz. simultaneous uptake of  $\text{H}^+$  or equivalent loss of  $\text{HO}^-$ ) with all 3 species [3]. The positive correlation between the ability of horseradish peroxidase to activate  $\text{H}_2\text{O}_2$  and the need to bind  $\text{H}^+$  with  $\text{F}^-$  [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  $\text{HO}_2^-$  (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  $\text{H}_2\text{O}_2$ ; 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  $\text{HO}_2^-$ , as observed with MP-8, into a proton-coupled reaction; this enables the enzyme to bind  $\text{H}_2\text{O}_2$  as  $\text{H}^+$  and the required  $\text{HO}_2^-$  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 ex-

ample, act as mediator for the overall transfer of  $\text{H}^+$  from  $\text{H}_2\text{O}_2$  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  $\text{GuaH}^+\text{HO}_2^-$  could increase the local concentration of  $\text{HO}_2^-$  required for coordination to  $\text{Fe}^{\text{III}}$  in the first step and/or facilitate the transfer of  $\text{H}^+$  to the uncoordinated O atom (to give  $\text{FeO}^{3+}$  and  $\text{H}_2\text{O}$ ) 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/ $\text{GuaH}^+$ ), and holoenzyme. Full experimental details and further discussion will be published elsewhere.

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