Studies on Horseradish Peroxidase in Dimethyl Sulphoxide/Water Mixtures

THE ACTIVATION OF HYDROGEN PEROXIDE AND THE BINDING OF FLUORIDE

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We studied the variation in spectra and in reactivity towards H_2O_2 of solutions of horseradish peroxidase in dimethyl sulphoxide/water mixtures, obtained by diluting stock solutions of the enzyme in either water or dimethyl sulphoxide, and assayed the enzymic activity and studied the binding of F^- by the peroxidase in 65% (v/v) dimethyl sulphoxide. A broadly similar pattern of changes is observed whether one starts from water or from dimethyl sulphoxide; the changes are essentially reversible, though hysteresis is observed. When the dimethyl sulphoxide content of the solvent mixture is increased, the peroxidase retains its ability to activate H_2O_2 up to 74% (v/v) dimethyl sulphoxide. The peroxidase in 65% (v/v) dimethyl sulphoxide binds F^- together with a proton (or the equivalent loss of HO^-), as already established for aqueous solutions. We point out that the occurrence in such solutions of both the ability to activate H_2O_2 and the inability to bind F^- without taking up H^+ or losing HO^- supports the proposed mechanism for activating H_2O_2 , whereby the protein binds the substrate in the form of the much more reactive HO_2^- .

We are interested in the role of the protein in modifying the thermodynamic and kinetic properties of iron porphyrins in enzymes such as the peroxidases (Pratt, 1975). We have studied the properties of horseradish peroxidase (EC 1.11.1.7) and the protein-free haemin in organic and aqueous organic solvents (Adams *et al.*, 1978; Collier *et al.*, 1979). We are here interested in the mechanism of activation of H_2O_2 by horseradish peroxidase.

Recent experimental results on the reactions of H_2O_2 with protein-free iron porphyrins have enabled comparisons to be made with peroxidases, catalases and myoglobin (all of which are haemoproteins) and have led to the proposal of a mechanism for the 'activation' of H_2O_2 by catalase and peroxidase enzymes at physiological pH (Pratt, 1975). Jones (1973) has also discussed possible mechanisms for the activation of H_2O_2 by catalase.

Protein-free iron porphyrins and myoglobin undergo reactions with H_2O_2 which appear to be analogous to, but much slower than, those of the enzymes. Like haemoglobin and myoglobin, these enzymes will bind a variety of simple anions such as F^- , CN^- and I^- , but, unlike the former, the enzymes always bind the anions together with a proton (or, conversely, with the loss of HO^-); for references see Pratt (1975) and Dunford & Stillman (1976). There appears to be no direct and quantitative

evidence (e.g. from competitive-inhibition studies) that F^- and other ions react at the same site on the enzyme as H_2O_2 . However, kinetic studies on the reaction of F^- , CN^- (both with a proton) and H_2O_2 with horseradish peroxidase and cytochrome *c* peroxidase as a function of pH revealed the existence of some ionizable (but unidentified) group on the proteins with identical p*K* values for the different reactions; and this can be taken as quantitative, though indirect, evidence that F^- , CN^- (and probably other anionic ligands) and H_2O_2 all react with the same site on the enzymes (Dunford & Stillman, 1976).

It appears therefore that the active sites in these enzymes share the unusual properties of (a) being able to activate H_2O_2 , but (b) being unable to bind simple anions except when a proton is also bound (or HO^- lost). Property (b) must represent either an intrinsic part of the mechanism for (a) or an additional and coincidental feature with no obvious physiological relevance. We have suggested (Pratt, 1975) that this curious effect of pH probably reflects the mechanism whereby the protein can activate H_2O_2 . If we write the binding of a very strong acid such as HI in the form of equilibrium (1) or (2) (where HRP represents horseradish peroxidase)

Abbreviation used: Me_2SO , dimethyl sulphoxide.

then the analogous equilibrium for the binding of a very weak acid such as H_2O_2 ($\text{p}K \sim 13$) can be written as in equilibria (3) or (4):

The protein can then 'activate' H_2O_2 (i.e. increase the rate constants for the reactions of H_2O_2) by binding the substrate according to the pH-independent equilibria (3) or (4) and thereby delivering the substrate to the active site in the form of the much more reactive nucleophile HO_2^- . It is perhaps significant that the catalytic activity of the protein-free iron porphyrin increases with pH and reaches the pH-independent value of the catalase enzymes at approx. pH 13 (Brown *et al.*, 1970).

We have been interested in obtaining further experimental evidence as to whether properties (a) and (b) above are closely linked, as required by the above theory. It seems reasonable to assume that properties (a) and (b) would each require the functional integrity of some structural feature of the protein near the active site (the iron atom). A comparative study of the effect on these two properties of gradual changes in the environment (e.g. in the composition of the solvent) might reveal whether they are linked or not. We have therefore been studying the properties of horseradish peroxidase in various organic solvents and report here our results on (1) the variation in spectra and reactivity towards H_2O_2 in Me_2SO /water mixtures and (2) the preservation of property (b) (as evidenced by the effect of pH on the binding of F^-) as well as property (a) in solvents containing at least 65% (v/v) Me_2SO .

For a recent review on peroxidases in general see Dunford & Stillman (1976). Previous work on horseradish peroxidase in Me_2SO /water mixtures, mainly at low temperatures, has been summarized by Douzou (1974). Some of our results relating to Fig. 1 have been reported in brief (Adams *et al.*, 1975). A recently published book provides useful information on Me_2SO and Me_2SO /water mixtures (Martin & Hauthal, 1976).

Materials and Methods

Solvents and reagents

Stock solutions of horseradish peroxidase (Miles Laboratories, Cape Town, South Africa) in water and Me_2SO and of haemin (BDH, Poole, Dorset, U.K.) in Me_2SO were prepared approximately once a month and stored in a refrigerator. Guaiacol (Riedel-de Haën, Hanover, W. Germany) was used without further purification. Me_2SO (Merck, Darmstadt, W. Germany) was purified by twice distilling 250 ml portions under vacuum (approx. 10 mmHg) and discarding the first and last 30 ml portions; the

solvent was stored over Linde 5A molecular sieve. Note: mole fraction of Me_2SO = (mol of Me_2SO in mixture)/(mol of Me_2SO + mol of water).

Enzyme assay

The enzymic activity of horseradish peroxidase was assayed at 25°C by following the rate of formation of the coloured tetraguaiacol from H_2O_2 and guaiacol (Chance & Maehly, 1955). From the observed rate in the presence of excess guaiacol one can calculate the rate constant (K_1) for the reaction of H_2O_2 with the Fe(III) form of the enzyme. The reaction mixture should contain 20 mM-guaiacol, 10 mM-potassium phosphate buffer, pH 7.0, 10 mM- H_2O_2 and approx. $0.1 \mu\text{M}$ -enzyme, and the formation of the yellow product is monitored at 470 nm. Observations on the application of this assay to solutions of the enzyme in 0.3 mole fraction Me_2SO are given in the Results section, paragraph (4).

Spectrophotometry

All measurements of spectra were conducted at $25.0 \pm 0.1^\circ\text{C}$ by using a thermostatically controlled cell holder in either a Pye-Unicam SP.1800 or a Jasco Uvidec-1 digital spectrophotometer.

pH measurements

These were carried out in 0.3 mole fraction Me_2SO with a Beckman glass electrode, which was equilibrated in the solvent for several hours before use. After each addition of reagent (HClO_4) the solution was stirred and then left for up to 10 min until a steady reading was attained.

Results

(1) Variation of spectroscopic properties of horseradish peroxidase in Me_2SO /water mixtures

Changes in the nature and environment of the iron porphyrin have been monitored by following changes in the region of the Soret band (400–410 nm) at 25°C as the solvent composition is altered. We have followed these changes in both directions by diluting an approx. 0.5 mM (or approx. 0.8 mM) stock solution of the enzyme in water (or Me_2SO) with the appropriate solvent mixture to give final concentrations of approx. $5 \mu\text{M}$ (or $3 \mu\text{M}$).

In all cases (except that of diluting the stock solution in water with water, where the initial spectrum remains unchanged) dilution gives an 'initial' spectrum, which is then converted into a 'final' spectrum. This interconversion (at least after dilution from stock solutions in water) shows isosbestic points and is kinetically biphasic, involving two consecutive first-order reactions; the first stage is fast ($t_{\frac{1}{2}}$ approx. 1 min), but the second stage is much slower ($t_{\frac{1}{2}}$ from 5 min up to 1 h or so). The final spectra show no further change over at least 12 h.

Table 1. Absorption spectra in the Soret-band region (400–410nm) exhibited by 'initial' and 'final' forms of horseradish peroxidase in Me₂SO/water mixtures

Relative and absolute values of $10^{-4}\epsilon$ were estimated to be correct to ± 0.05 and ± 0.1 respectively, and relative and absolute values of λ to ± 0.3 and ± 0.5 nm respectively.

Mole fraction of Me ₂ SO	10 ⁻⁴ × ε _{403.5}		λ _{max.} (nm) Final	Reactivity towards H ₂ O ₂	
	Initial	Final		Initial	Final
(a) Dilution from stock solution in water					
0.00	9.10	9.10	403.5	+	+
0.03	9.45	9.45	403.5	+	+
0.05	9.65	9.65	404.2		
0.07	9.91	9.91		+	+
0.10	10.32	10.32	405.2		
0.11		10.25			
0.16	10.35	10.82	406.0		
0.17	10.74	10.74		+	+
0.20	10.61	11.11			
0.30	11.15	11.42		+	+
0.30	11.20	11.70	406.5		
0.34	11.26	11.65	407.0		
0.375	11.30	12.03		+	+
0.38	11.30	11.89		+	+
0.40	11.25	11.90	405.8	+	+
0.425	11.38	14.35	404.4	+	—
0.44	11.45	14.70	404.2	+	—
0.50	10.76	14.81	404.3	?	—
0.50	10.76	15.01			
0.53	16.15	14.50	404.4	—	—
0.56	15.35	13.50			
0.61	15.25	13.20	404.4	—	—
0.63	14.95	12.86		—	—
0.64	14.81	12.59	404.4	—	—
0.86	13.77	11.11	404.6	—	—
(b) Dilution from stock solution in Me ₂ SO					
0.00		9.00	403		+
		8.78			
0.10		11.08	404		+
		10.65			
0.20		11.02	405		+
		12.03			
0.25		12.16			+
0.30		14.84	403		—
		16.26			
0.40		16.23	403.5		—
		16.23			
0.50		15.90	403.8		—
0.60		13.67			—
0.70		11.65	403.8		—
0.80		10.39			—
1.00		8.81			—

The results obtained by diluting a stock solution of the enzyme in water are summarized in Table 1(a) and the more limited data obtained with stock solutions in Me₂SO in Table 1(b). The rapidity of the first step in the interconversion prevented a determination of the wavelength of the Soret band in the initial forms. The wavelength (403.5nm) and molar absorption coefficient (9.1×10^4) of the Soret band of

horseradish peroxidase in water agree well with the published values of 403nm and 8.95×10^4 – $9.10 \times 10^4 \text{M}^{-1} \cdot \text{cm}^{-1}$ (Maehly, 1955).

(2) Variation in reactivity towards H₂O₂

Since the presence of the protein appears to enhance the rate of reaction of the Fe(III) ion in horseradish peroxidase with H₂O₂ without markedly

affecting the rates of the subsequent reactions with added reducing agents (Pratt, 1975), we have used the rate of reaction with H_2O_2 as a quick and convenient means of testing for probable enzymic activity in different solvent mixtures. H_2O_2 reacts with the Fe(III) form of the enzyme in aqueous solution to give the so-called Compound I, which contains two oxidizing equivalents per Fe atom above that of Fe(III). Even in the absence of added reducing agents, Compound I is rapidly reduced by groups present in the enzyme ('endogenous donors') to Compound II, which contains one additional oxidizing equivalent per Fe atom and which is relatively stable in the absence of added reducing agents. Treating the active enzyme in water with H_2O_2 alone therefore produces Compound II, which is characterized by an absorption band at 418 nm (Maehly, 1955).

Qualitative tests were made to determine whether the initial and final species in each solvent mixture reacted rapidly with approx. 0.1 mM- H_2O_2 . To test the reactivity of the final species, H_2O_2 was added after the final spectrum had been attained. To test the reactivity of the initial species, further experiments were carried out in which the stock solution was diluted with a solvent mixture containing H_2O_2 . The results of these tests in different solvent mixtures are also given in Table 1.

The tests serve clearly to distinguish between forms of the enzyme which are reactive (reaction over 90% complete within 10s) and inert (less than 10% reaction observed over 10min). Assuming that all the rates are first order with respect to iron porphyrin and that there are no further complications, then this indicates a difference of at least 10^3 in rate between the active and inactive forms. In all cases where reaction with H_2O_2 occurred at a significant rate, the spectra produced were very similar; we conclude that all these reactions give the same product with the same high (approx. 100%) degree of reaction, independent of the solvent composition. No spectra were observed that could be attributed to partial reaction, i.e. to different isoenzymes becoming inactivated at different solvent compositions. There was no obvious evolution of gas bubbles at any solvent composition, i.e. there was no change from peroxidase to catalase activity at any point.

(3) Composite picture of variation in spectroscopic properties and reactivity towards H_2O_2

The composite picture of the final values of the molar absorption coefficients obtained by dilution from both water and Me_2SO , together with the results of the tests for reactivity with H_2O_2 , is given in Fig. 1.

The plot in Fig. 1 shows that a broadly similar pattern of changes is observed, whether one starts from water or from Me_2SO , but that the greatest

Fig. 1. Variation of absorption spectra and reactivity of horseradish peroxidase in Me_2SO /water mixtures. Composite picture of the variation in spectra (molar absorption coefficient at 403.5 nm) and reactivity towards H_2O_2 of solutions of peroxidase in Me_2SO /water mixtures, when prepared by dilution from stock solutions in water ($\bullet$, $\circ$), or Me_2SO ($\blacksquare$, $\square$). Filled circles and squares denote forms that react rapidly with H_2O_2 . Data are taken from Table 1.

difference occurs in the range of 0.25–0.40 mole fraction Me_2SO . The sharp discontinuities at 0.25 and 0.40 mole fraction suggest that, in this region at least, all the isoenzymes show similar behaviour.

To check the existence of hysteresis, we carried out the following experiments to examine the effect of changing the direction in which we alter the solvent composition on the activity of the enzyme as measured by the rate of reaction with H_2O_2 . The numbers below give the mole fraction of Me_2SO in the solvent mixture, and the activity towards H_2O_2 is indicated in parentheses. At each stage of each experiment the solution was divided, one part being treated with H_2O_2 and the other part being diluted with the appropriate solvent to give the next solution:

- (1) 0.0 (i.e. stock in water) $\rightarrow$
0.4 (active) $\rightarrow$ 0.3 (still active)
- (2) 0.0 $\rightarrow$ 0.5 (inactive) $\rightarrow$ 0.35 (inactive) $\rightarrow$
0.2 (active)
- (3) 1.0 (i.e. stock in Me_2SO) $\rightarrow$
0.2 (active) $\rightarrow$ 0.35 (still active)

As regards reactivity towards H_2O_2 , it appears that if we start from water we can go forward and backward along the solid line in Fig. 1, provided that we do not go beyond 0.4 mole fraction. If we go beyond 0.4 mole fraction and then reverse, we now follow the broken line. Conversely, if we start from Me_2SO

and go below 0.25 mole fraction and then reverse, we now follow the solid line. The hysteresis is obviously genuine.

(4) Enzyme assay

In order to demonstrate that the observed variation in reactivity towards H_2O_2 does indeed parallel the variation in the overall enzymic activity, we have assayed the activity of the active and inactive (towards H_2O_2) forms of horseradish peroxidase in 0.3 mole fraction Me_2SO , by using the guaiacol method (see the Materials and Methods section); a comparison of these two forms will provide an internal check on the applicability of the assay method. The application of the usual purely aqueous assay method to aqueous organic solvents is bound to be affected to a considerable, but unknown, extent by the change in solvent composition.

On using the assay in 0.3 mole fraction Me_2SO it was found that the yellow colour was generated, but that a precipitate formed which prevented an accurate spectrophotometric determination of the absorption coefficient. Formation of the precipitate was avoided by omitting the buffer, but the yellow colour was then discharged much more rapidly. We conclude that in 0.3 mole fraction Me_2SO the assay is adequate as a qualitative test for enzymic activity, but that it will only give a minimum value for K_1 , and that, in the absence of further work on the kinetics of formation and destruction of the yellow product in this solvent, it cannot serve as a quantitative measure of enzymic activity.

The results of assays on various solutions are given in Table 2 and can be summarized as follows. (1) Our values for K_1 in water at 25°C (1.0×10^7 and $1.1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) compare reasonably well with the published values of $0.89 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 20°C and $1.03 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 30°C (Chance & Maehly, 1955). (2) The assay does work, at least qualitatively, in 0.3 mole fraction Me_2SO and the results parallel those obtained for reactivity towards H_2O_2 ; one form is enzymically active and its activity is at least 5×10^3 times greater than that of the 'inactive' form. (3) As expected, horseradish peroxidase in

pure Me_2SO shows no enzymic activity; but, since we do not know whether the assay would work in this solvent, this result is not meaningful. (4) Dilution of a fresh stock solution of the enzyme in Me_2SO to almost 100% water (actually 0.067% Me_2SO) regenerates at least 45% of the activity expected for the native enzyme in water. Failure to regain full activity could mean either (i) that some of the enzyme (for example, certain isoenzymes) had been irreversibly denatured while the remainder regained full activity, or (ii) that none of the enzyme had been irreversibly destroyed, but had a modified structure (owing, for example, to the inclusion of Me_2SO molecules) with lower activity. The slightly different spectrum (see Table 1) and the slow increase in activity observed over 2–180 min, which could be due to the release of Me_2SO molecules, suggests possibility (ii) rather than (i).

(5) Binding of F^- by active horseradish peroxidase in 0.3 mole fraction Me_2SO

Preliminary tests in 0.3 mole fraction Me_2SO of the interaction of active peroxidase with anions which are known to be bound together with the uptake of a proton (or loss of HO^-) in water (F^- , Cl^- , I^- , N_3^- , HCO_2^- , CN^-) indicated that F^- would be the most convenient to study. As expected, the addition of increasing amounts of aqueous HClO_4 (i.e. an acid with a non-co-ordinating anion) merely changed the spectrum to that of protein-free haemin (bands at 404.5, 500 and 625 nm) with no evidence for the formation of any intermediate compound. The addition of a solution of KOH in 0.3 mole fraction Me_2SO had no significant effect on the spectrum up to at least pH 8.5. If one assumes that the pK for the formation of the hydroxo form of peroxidase in aqueous solution is 11 or over (Dunford & Stillman, 1976), then we can conclude that the sixth ligand present in the enzyme over the range to be investigated is a neutral ligand (water or Me_2SO) or is absent [i.e. the Fe(III) ion is five-co-ordinated].

The pK of HF in Me_2SO /water mixtures has not been reported, though it is expected to be considerably higher than in water (where $\text{pK} = 3$) because the

Table 2. Enzymic assay of solutions of horseradish peroxidase in Me_2SO /water mixtures
 Me_2SO stock solution was 12 h old. —, No observed activity ($K_1 < 4 \times 10^4$).

Stock solution	Mole fraction of Me_2SO after dilution	Time (min) after dilution	Rate constant, K_1 ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Water	0.0	10	$\begin{cases} 1.08 \times 10^7 \\ 1.02 \times 10^7 \end{cases}$
Water	0.3	10	$\geq 2 \times 10^5$
Me_2SO	1.0	10	—
Me_2SO	0.3	10	—
Me_2SO	0.0 (0.067%)	2	2.4×10^6
		30	3.3×10^6
		180	4.6×10^6

decrease in hydrogen-bonding increases the activity of the anion (Martin & Hauthal, 1976). The pK of HF in 0.3 mole fraction Me_2SO was determined from a potentiometric titration of 50 ml of an 0.5 M solution of KF in 0.3 mole fraction Me_2SO with aqueous 1.2 M HClO_4 at 25°C by using a glass electrode (see the Materials and Methods sections). The mid-point of the titration curve corresponded to pK 8.75.

If we now determine the apparent equilibrium constant K_{app} , where

$$K_{\text{app}} = \frac{[\text{Fe}^{\text{III}}\text{F}^-]}{[\text{Fe}^{\text{III}}](\text{HF}) + [\text{F}^-]}$$

in a pH region where $\text{pH} < pK$ and the added fluoride is present almost entirely as the undissociated HF, then $\log K_{\text{app}}$ will be virtually independent of pH (except for relatively small changes in the degree of ionization of HF, small changes in ionic strength, perhaps specific binding of buffer anions to the polypeptide etc.) if the binding of F^- by peroxidase involves a proton, but will increase approximately linearly with pH if the enzyme binds F^- alone.

Values of $\log K_{\text{app}}$ for the co-ordination of F^- by 4.8 μM solutions of the active form of horseradish peroxidase in 0.3 mole fraction Me_2SO were determined at pH 6.45, 6.89 and 7.46, i.e. below the pK of HF. Phosphate buffers were used and adjusted to the desired pH by the addition of small amounts of concentrated aqueous HClO_4 . The buffering capacity was limited by the lower solubility of phosphate in this solvent, and the pH quoted in Table 3 is therefore the initial pH. The concentration of fluoride was varied by adding small known volumes of 0.04 M- and 0.2 M-KF in the same solvent; excess of solid KF was finally added to obtain the spectrum of the fully formed species. Changes in the spectrum were followed over the region 360–650 nm. The initial spectrum showed bands at 406.5, 502 and 637.5 nm. Equilibrium was established 'instantaneously' after each addition of reagent, isosbestic points were observed at 388, 426, 473, 546 and 649 nm, and the final spectrum showed bands at 405, 494 and 616 nm and remained unchanged for at least 12 h. We are therefore dealing with only a single, rapidly esta-

Fig. 2. Equilibrium constant for the binding of F^- by horseradish peroxidase in 0.3 mole fraction Me_2SO . Evaluation of experimental data for the binding of F^- by the active form of peroxidase in 0.3 mole fraction Me_2SO at pH 6.45 ($\circ$), pH 6.89 ($\square$) and pH 7.46 ($\triangle$).

blished equilibrium. Values of $\log K_{\text{app}}$ were determined from changes in the $A_{407.5}$, corrected for the small changes in volume. The plots of $\log[A_x - A_{\text{initial}}]/(A_{\text{final}} - A_x)$ against $\log([\text{HF}] + [\text{F}^-])$ are shown in Fig. 2 and the results are given in Table 3. The values of the slope $n = 1.0$ –1.1 show that only one F^- is being co-ordinated, and the absence of any significant variation in $\log K_{\text{app}}$ with pH shows that one proton is also involved in the equilibrium.

Discussion

We have studied the variation in spectra and in reactivity towards H_2O_2 of solutions of horseradish peroxidase in Me_2SO /water mixtures over the whole range of composition and have also assayed the enzymic activity and studied the binding of F^- by the enzyme in 0.3 mole fraction Me_2SO . The spectroscopic studies have been focused on changes in the 400–410 nm region (Soret band), which are due to transitions within the cofactor (the iron porphyrin) and hence provide a useful indicator of changes at or near the active site (the iron atom). Qualitative studies of reactivity towards H_2O_2 provide an easy

Table 3. Binding of F^- by horseradish peroxidase in 0.3 mole fraction Me_2SO

n was obtained from slope of plots in Fig. 2, and $\log K_{\text{app}}$ from reciprocal of $([\text{HF}] + [\text{F}^-])$ at 50% formation in Fig. 2.

pH	Value of n	$\log K_{\text{app}}$
6.45	1.03	3.74
6.89	1.06	3.62
7.46	1.11	3.57

means of distinguishing between 'active' and 'inactive' forms of peroxidase, which differ in their rate of reaction with H_2O_2 by at least 10^3 . We have found that the enzymic assay using guaiacol can be applied qualitatively, but not quantitatively, in 0.3 mole fraction Me_2SO , and that the assay confirms the division into 'active' and 'inactive' forms, based on reactivity towards H_2O_2 . No evidence was found for catalase activity under any conditions or for significant differences in reactivity between the different isoenzymes (Dunford & Stillman, 1976) present in the sample of horseradish peroxidase.

The experimental data given in Table 1 and Fig. 1 show that varying the solvent composition in Me_2SO /water mixtures has a marked effect on both the spectra and the reactivity of horseradish peroxidase, and that the changes are essentially reversible but show hysteresis. If the rate of equilibration is slow (as found with this enzyme), then one may expect to observe hysteresis if one does not vary the conditions (e.g. temperature or, as here, solvent composition) slowly enough to enable the new equilibrium to be established at every point along the line (Tanford, 1968). Hysteresis has, in fact, been reported in the reversible denaturation of various polypeptides and polynucleotides on varying the temperature (Douzou, 1974), but the present example appears to be the first reported case of hysteresis in the reversible denaturation of an enzyme. It provides the unique opportunity of being able to compare active and inactive (reversibly denatured) forms of the same enzyme under identical conditions. The retention of activity right up to 0.4 mole fraction (and even 0.5, with the 'initial' forms prepared by dilution from water) and the (at least partial) recovery of activity after dissolution in 100% Me_2SO deserve note. As Tanford (1968) comments: 'Dimethyl sulphoxide is ... a markedly ineffective denaturing agent.'

The breaks in the curves of absorption coefficient with solvent composition at 0.25 and 0.4 mole fraction for the 'final' forms obviously reflect a significant change in structure between the active and inactive forms. The complete discontinuity between the active and inactive initial forms obtained by dilution from water is also noteworthy. Parallel spectroscopic studies on haemin in Me_2SO /water mixtures (Collier *et al.*, 1979) show that the haemin in the 'inactive' form cannot be 'free', but must remain bound to the protein in some way. Since these breaks are observed in the absence of any added electrolyte, they cannot be ascribed to changes in, for example, ionic strength or the activities of added electrolytes, but must be caused directly by changes in solvent composition (including any changes in the activities of water, Me_2SO , H^+ and HO^-).

Within each family of 'active' forms (initial and final forms prepared by dilution from water, and

final forms from Me_2SO ; we have not investigated the initial forms from Me_2SO) we observe a continuous and much more gradual change. Change in solvent composition is expected to alter the spectrum either directly by affecting the co-ordinated ligand (replacement of water by Me_2SO , change in the degree of hydrogen-bonding etc.) or the interaction of the porphyrin with the solvent, or indirectly by affecting the structure of the protein. The movement of the Soret band (first to longer and then to shorter wavelength, as the Me_2SO content increases) in the final forms derived from water (see Table 1) shows that in this case at least there is more than one factor involved. The roles of the different factors cannot at present be distinguished.

These studies have greatly extended the range of conditions under which the binding of anions and protons by the active enzyme can be studied. Horseradish peroxidase retains its activity up to 0.4 mole fraction (74%, v/v) Me_2SO , where the environment of the protein is very different from that in aqueous solution. The change in the wavelength and intensity of the Soret band in the absorption spectrum indicates that the iron porphyrin, which includes the active site, has been affected by the change in environment. The experimental results in section (5) show that in 0.3 mole fraction Me_2SO (i.e. the same solvent composition as was used for the enzymic assay) the peroxidase reversibly binds one F^- with the uptake of one H^+ (or loss of one HO^-).

We have therefore shown that horseradish peroxidase retains both (a) the ability to activate H_2O_2 and (b) the inability to bind F^- (and presumably other simple anions) without simultaneously binding a proton (or losing HO^-) under conditions significantly different from those obtaining in aqueous solution. This provides circumstantial evidence that property (b) is linked to (a) and hence additional experimental evidence that the peroxidase (and catalase) enzymes activate H_2O_2 by binding the substrate in the form of the much more reactive anion HO_2^- according to eqn. (3) or (4). Further speculation as to the mechanism is justified only if there is some evidence to indicate which of eqns. (3) or (4) correctly represents the activation of H_2O_2 .

It has generally been assumed that horseradish peroxidase exists in neutral aqueous solution as the Fe(III) aquo complex. The only evidence was the observation of a pH-dependent equilibrium (pK 11), which appeared to be analogous to the equilibrium (pK 9) between the aquo and hydroxo complexes of metmyoglobin. However, the demonstration (Epstein & Schejter, 1972) that the pK observed for the peroxidase cannot represent the simple equilibrium between the aquo and hydroxo complexes means that we now do not know whether the axial ligand is water, HO^- or some other weak base in its unprotonated form, or even whether there is any

sixth ligand at all. We hope to obtain further information on the co-ordination of the Fe(III) ion in horseradish peroxidase.

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