Studies on the Equilibria and Kinetics of the Reactions of Peroxidases with Ligands. I. The Reaction of Ferroperoxidases with Carbon Monoxide*

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ABSTRACT: The equilibrium and kinetics of the reaction of horseradish and fig ferroperoxidases with carbon monoxide have been studied. For both peroxidases, at 20°, the equilibrium constant (L) is about 4.5×10^6 M^{-1} and the combination velocity constant (l') about 4×10^3 M^{-1} sec^{-1}.

The same value of the combination velocity constant is obtained with rapid mixing as with flash photolysis at widely different CO concentrations. Carbon monoxide is displaced by ferricyanide at a rate much higher than that calculated from the equilibrium constant and the "on" constant.

Peroxidases are a heterogeneous group of heme proteins which may act as catalysts in reactions between peroxides and an electron donor (Paul, 1963; Nichols, 1962; Saunders *et al.*, 1964). The ferri form of peroxidase is thought to combine with peroxides to give a series of complexes which in turn react with the oxidizable substrates (Keilin and Mann, 1937; Theorell, 1941; Chance, 1951).

Heretofore only minor attention has been paid to the reactions of the ferro form of peroxidases with ligands, although it has been suggested that ferroperoxidase is involved in the oxidative and hydroxylating activity of this enzyme (Nichols, 1962; Lemberg and Legge, 1949; Yamazaki and Piette, 1963; Klapper and Hackett, 1963; Mason, 1958).

On addition of strong reducing agents the ferriperoxidase changes to the ferro derivative, the spectrum of which is very similar to that of ferromyoglobin or ferrohemoglobin. Unlike the latter proteins, however, ferroperoxidase is unstable in the presence of molecular oxygen, reverting to the ferri compound. With other ligands, *e.g.*, CO, NO, CN, and RCN, ferro horseradish peroxidase gives additional compounds very similar to those of hemoglobin and myoglobin (Keilin and Hartree, 1951).

In view of this behavior it was decided to study more closely the equilibria and kinetics of the reactions of ferroperoxidase with various ligands. Such studies would provide a comparison between the reactions of ferroperoxidase with those of ferrohemoglobin and myoglobin. Horseradish peroxidase and similar plant peroxidases, just like myoglobin, contain only one heme per molecule and offer an opportunity for studying the specific effect of the apo protein on the reactivity of the heme with a given ligand.

In the present paper the reaction of horseradish peroxidase and of a similar protein extracted from fig tree latex with carbon monoxide is described. Similar studies with other ligands will be reported later.

Materials and Methods

Peroxidases. The horseradish peroxidase (HRP)[1] used was a commercial product obtained from Boehringer (POD I lot 6433504). Fig peroxidase (FP) was prepared according to M. El-Fekih and D. Kertesz (manuscript in preparation). Like HRP, FP contains one protoheme per molecule; its catalytic activities and the absorption spectra of its derivatives are essentially identical with those of HRP.

The preparations of both peroxidases used in the present experiments were homogenous in the ultracentrifuge. The peroxidases were stored under ammonium sulfate; before use they were dissolved in water and the solutions were dialyzed against water to remove the ammonium sulfate.

Concentrations of the various derivatives of the peroxidases were determined spectrophotometrically using the following values of the extinction coefficients: ferro derivative, ϵ_{mM} (at 438 mμ) 74.5; CO derivative, ϵ_{mM} (at 423 mμ) 135.0 (Keilin and Hartree 1951).

CO Equilibrium. The equilibrium of ferroperoxidases with CO was determined in the following way. A dilute solution of peroxidase ($0.7–3 \times 10^{-6}$ M) was introduced into a 1-cm optical cuvet closed by a vaccine cap at the top and having a volume of 3.95 ml. The cuvet contained a small glass bubble, to allow for mixing. Care was taken to avoid air bubbles in the cuvet. Addi-

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[1] Abbreviations used in this work: HRP, horseradish peroxidase; FP, fig peroxidase.

TABLE I: Velocity Constant (l') for Combination of Carbon Monoxide with Reduced HRP and FP as Obtained by Stopped-Flow Measurements.[a]

	pH 7.0			pH 9.18		
	l' (M^{-1} sec^{-1})		ΔH^*	l' (M^{-1} sec^{-1})		ΔH^*
	12.5°	20.0°	(kcal)	12.5°	20.0	(kcal)
HRP	2.3×10^3	3.4×10^3	8	3.1×10^3	4.5×10^3	8
FP	1.5×10^3	2.5×10^3	11	1.9×10^3	3.0×10^3	10

[a] Experiments were carried out in 0.1 M phosphate buffer, pH 7.0, and 0.025 M borate, pH 9.18. At every temperature and pH the reaction was followed at λ 438 mμ.

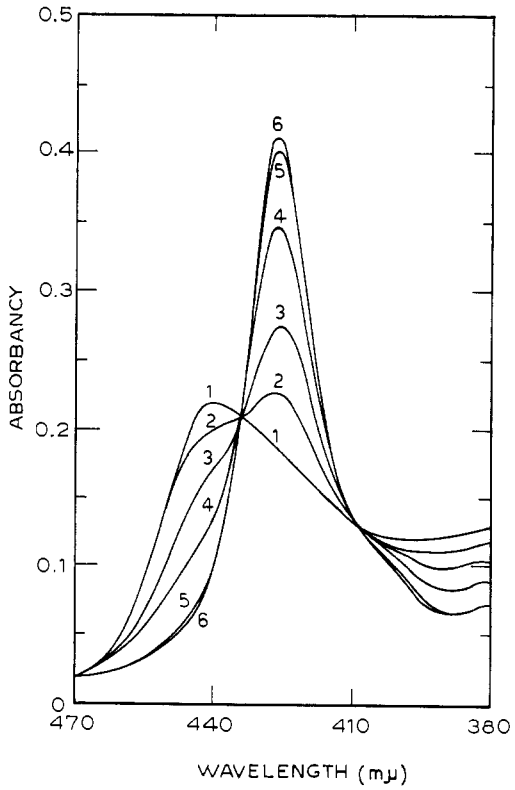

FIGURE 1: Equilibrium between HRP and CO at 20° in 0.05 M borate, pH 9.18. HRP concentration = 2.82×10^{-6} M. The total concentrations of CO (free and bound) are the following: 1 = 0; 2 = 0.635×10^{-6} M; 3 = 1.27×10^{-6} M; 4 = 2.54×10^{-6} M; 5 = 3.81×10^{-6} M; 6 = 6.35×10^{-6} M.

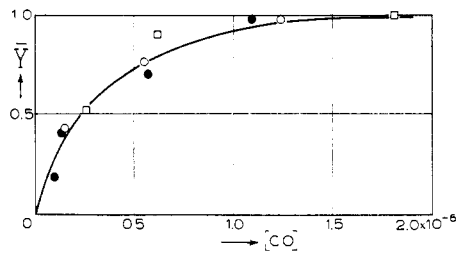

FIGURE 2: Equilibrium curve for HRP and CO at 20°. The notations are as follows: ○, results at pH 7.0, 0.1 M phosphate, HRP concentration = 2.62×10^{-6} M; ●, results at pH 9.18, 0.05 M borate, HRP concentration = 2.82×10^{-6} M; □, results at pH 7.0, 0.1 M phosphate, HRP concentration = 0.72×10^{-6} M. The curve shown is a rectangular hyperbole corresponding to $L = 4.5 \times 10^6$ M^{-1}.

tions of solutions to the cuvet were made from a syringe with the needle inserted in the vaccine cap; another needle appropriately inserted through the cap served for the outflow of excess liquid.

The ferriperoxidase solution was reduced by adding ~0.02 ml of a 1.5% solution of sodium dithionite in 0.1 M Na_2HPO_4, prepared anaerobically. The formation of the derivative was followed spectrophotometrically. The ferroperoxidase so obtained was then titrated with a 0.5×10^{-3} M solution of CO (water equilibrated with 1/2 atm of CO at 20°). The additions (5–50 μl) were made with an "Agla" microsyringe. The dilution effect was ~1%. After each CO addition the solution was placed in a thermostated cuvet holder and, after the solution had reached temperature equilibrium, the spectrum was recorded; from these spectra and from those obtained in the absence and in the presence of a great excess of CO the fractional saturation corresponding to each CO addition was obtained. The concentration of free CO was calculated by subtracting the CO bound from the total CO added.

The optical density measurements were made in a Beckman DK-1 recording spectrophotometer and at a temperature of 30 or 20 ± 0.2°.

Kinetic Experiments. Measurements of fast reaction rates were made with a Gibson stopped-flow apparatus (Gibson and Milnes, 1964) equipped with a 2-cm observation tube. The solutions of ferroperoxidase to be used in the stopped-flow experiments were prepared in a tonometer by the addition of small amounts of dithionite to degassed solutions of ferriperoxidase.

Slow reactions (half-time greater than a few seconds) were followed in the Beckman DK-1 recording spectro-

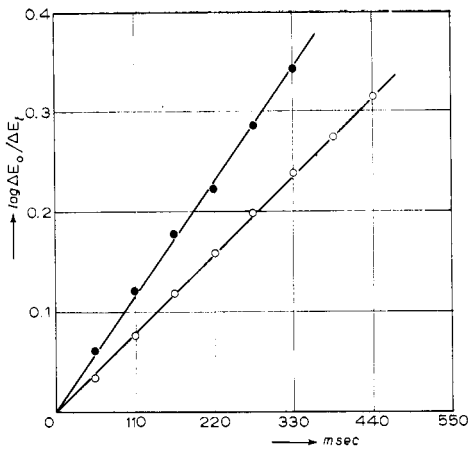

FIGURE 3: Time course of the combination of CO with reduced HRP at pH 7.0 in 0.1 M phosphate (○) and pH 9.18 in 0.05 M borate (●) at 20°. Observations made at 423 mμ. Initial concentration of HRP = 2.31×10^{-6} M; initial concentration of CO = 0.5×10^{-3} M.

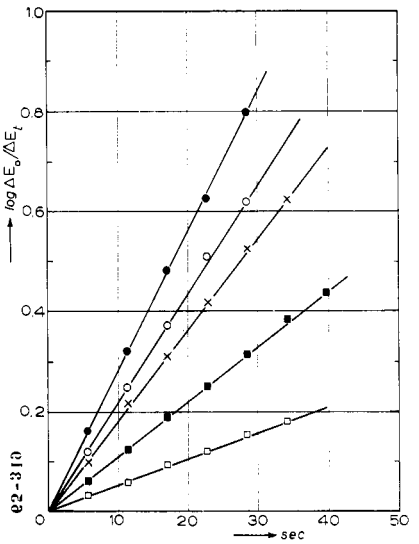

FIGURE 4: Combination of HRP with CO (at different concentrations), after photochemical dissociation, in 0.1 M phosphate buffer at pH 6.32 and 20°. Initial HRP concentration = 3.2×10^{-6} M; initial CO concentrations: □, 6.35×10^{-6} M; ■, 12.7×10^{-6} M; ×, 19×10^{-6} M; ○, 25.8×10^{-6} M; ●, 31.7×10^{-6} M. Observations made at 423 mμ.

photometer in the same type of cuvet used for the equilibrium measurements.

Flash Photolysis Experiments. These experiments were performed in the following way. A CO–peroxidase solution containing a known amount of free CO was prepared as described for the equilibrium measurements. The solution was brought to constant temperature in the spectrophotometer and its spectrum recorded. The cuvet was then quickly removed from the instrument, exposed briefly to the unfiltered light of a photographic flash or of a 250-w tungsten lamp, and transferred back into the spectrophotometer, and the change in optical density corresponding to the return to equilibrium in the dark was recorded. Under the conditions used more than 80% of the bound CO was photodissociated.

Results

Since both HRP and FP contain only one heme per molecule, the reaction of these proteins with CO should correspond to the following simple scheme

$$\text{ferroPer} + \text{CO} \underset{l}{\overset{l'}{\rightleftharpoons}} \text{ferroPer–CO} \qquad \text{(A)}$$

$$L = l'/l$$

where L is the equilibrium constant, and l' and l are the combination and dissociation velocity constants, respectively.

Equilibrium between Ferroperoxidase and CO. Figure 1 gives, as an example, the spectroscopic observations involved in one determination of the CO equilibrium of HRP. The equilibrium behavior for HRP under different experimental conditions is shown in Figure 2; the points lie on a rectangular hyperbola within experimental error, corresponding to the simple equilibrium of scheme A. This means that the value of n in the Hill equation, which is commonly used to describe the equilibria of heme proteins with ligands, is 1. The value of L corresponding to the full curve of Figure 2 is 4.5×10^6 M^{-1}. Two experiments on FP at 30° in 0.1 M phosphate buffer gave a value of $L = 3 \times 10^6$ M^{-1}.

The close agreement between the results of the equilibrium of HRP at different protein concentrations (Figure 2) is a good test of the validity of the procedure used in the determination of the equilibrium curves, since the errors involved in the determination of free CO depend on the protein concentration. It will be noticed that the value of L is practically independent of pH between 7 and 9.18.

Kinetics of Combination of CO with Ferroperoxidase. A. STOPPED-FLOW EXPERIMENTS. In these experiments the ferroperoxidase was made to react with a great excess of carbon monoxide, the concentration of the protein being 2.35×10^{-6} M and that of CO varying from 0.5 to 5.0×10^{-4} M. Under these conditions the time course of the reaction conformed to a pseudo-first-order process (Figure 3), the rate being proportional to the CO concentration. The value of l', the combination velocity constant, was obtained by the equation

$$l' = r(1/[\text{CO}]) \qquad (1)$$

where r is the observed pseudo-first-order rate constant $= \text{d} \ln (\Delta E_0/\Delta E_t)/\text{d}t$ and [CO] the total CO concentration. Equation 1 presupposes that the contribution of

the back reaction to the value of r is negligible; this is justified by the high value of the equilibrium constant.

The values of l' obtained in this way for HRP and FP are reported in Table I. It will be seen that the combination velocity constant for CO is similar for the two peroxidases. For both of them it changes little, though significantly, with pH (Figure 3).

The activation energy, ΔH^*, may be calculated from the effect of temperature on l'. For both proteins, and at pH 7 and 9, it has a value of about 9 kcal.

B. FLASH PHOTOLYSIS EXPERIMENTS. Figure 4 shows the time course of combination of CO with ferroperoxidase after photodissociation. In Figure 5 the initial pseudo-first-order rates obtained in experiments of this kind are plotted *vs.* the initial CO concentration. The slope of the line should give the value of l' and its intercept with the ordinate axis that of l. The values of l' obtained in this way for HRP and FP under different conditions are reported in Table II. They are very close

TABLE II: Velocity Constant (l') for the Combination of Carbon Monoxide with Reduced HRP and FP as Obtained from Flash Photolysis Experiments.[a]

HRP			FP		
pH	$l' \times 10^{-3}$ (M^{-1} sec^{-1})	Temp (deg)	pH	$l' \times 10^{-3}$ (M^{-1} sec^{-1})	Temp (deg)
5.13	2.0	20	7.0	1.7	20
6.32	2.1	20	7.0	2.5	30
7.0	2.3	20			
8.2	3.4	20			
9.18	3.5	20			
9.85	4.0	20			

[a] Experiments were carried out in 0.1 M phosphate buffer and 0.05 M borate. The reaction was followed at 423 mμ.

to those obtained in the stopped-flow experiments, in spite of the fact that the concentration range of CO in the latter case is orders of magnitude higher. The intercepts of the plots are very near to zero. This makes values of l obtained in this way highly uncertain; however, the data indicate that the constant is lower than 0.01 sec^{-1}.

The Dissociation of Carbon Monoxide from CO–Ferroperoxidase. Measurement of the rate of CO dissociation from CO–ferroperoxidase by the reaction with ferricyanide was attempted. This procedure rests on the assumption that only the nonliganded derivative can react with ferricyanide and that the whole reaction occurs according to the scheme

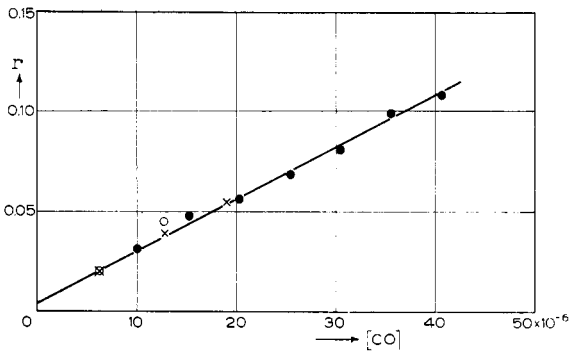

FIGURE 5: Initial apparent rate in sec^{-1} for the approach to equilibrium (after photodissociation of CO) *vs.* initial CO concentration for FP at 30°, in 0.1 M phosphate, pH 7.0. Different symbols indicate different experiments.

$$\text{ferroPer–CO} \rightleftharpoons \text{ferroPer} + \text{CO} \qquad \text{(B)}$$

$$\text{ferroPer} + \text{ferricyanide} \rightleftharpoons \text{ferriPer} + \text{ferrocyanide}$$

The experiments were performed in the following way. A solution of CO-ferroperoxidase (2.9×10^{-6} M) equilibrated with CO at low concentration (25×10^{-6} M) was mixed with a degassed solution of ferricyanide (final concentration 2×10^{-3} M). The reaction was followed in the spectrophotometer at 578 mμ.[2] The reaction followed simple first-order kinetics. The rate constants calculated at different pH values are included in Table III.

TABLE III: Dissociation Velocity Constant Obtained by Reaction of CO-HRP with Ferricyanide at 20°.[a]

pH	l (sec^{-1})
5.13	0.05
6.32	0.04
7.0	0.035
9.18	0.06
9.85	0.04

[a] Experiments carried out in 0.1 M phosphate and 0.05 M borate buffers.

Discussion

The shape of the equilibrium and kinetic curves for the reaction of ferroperoxidase with CO correspond to

[2] Since at such protein concentrations the optical density change in the visible region is very small, the transmission scale 90–100% had to be used to record the reaction. The absorption by ferricyanide prevented following the reaction in the Soret region, and higher peroxidase concentrations could not be used.

TABLE IV: Comparison of the Equilibrium Constant and the Combination and Dissociation Velocity Constants for Different Heme Compounds.[a]

	$L \times 10^{-6}$ (M^{-1})	$l' \times 10^{-5}$ (M^{-1} sec^{-1})	l (sec^{-1})	Solvent	Reference
Protoheme	0.4	160	40[b]	NaOH 0.02 N	Smith (1959) Wang (1962)
Monopyridine protohemochromogen	—	4.2		NaOH 0.02 N	Smith (1959)
Horse myoglobin	39	5.0	0.017	Phosphate, pH 7.0	Antonini (1965)
Horseradish peroxidase	4.5	0.045	0.001[b]	Borate, pH 9.18	This paper

[a] At 20°. [b] Values calculated from the equilibrium and the combination velocity constants.

the simple case of a protein having a single binding site, *i.e.*, to the reaction given in scheme A. The two peroxidases studied here, HRP and FP, give very similar results both as to the equilibrium and kinetics, although the small differences in the rates of combination with CO are certainly significant and reflect chemical differences between the two proteins.

Keilin and Hartree (1955) found that the affinity of HRP for CO was about 2500 times greater than that for cyanide. They give a value for the affinity constant for cyanide of 0.77×10^3 M^{-1}. From these data the affinity constant of ferroperoxidase for CO may be calculated to be about 2×10^6 M^{-1} at room temperature. This may be compared with the value of 4.5×10^6 M^{-1} found by us at 20° in direct determinations. In view of the relatively large errors involved in these measurements the agreement is satisfactory. The velocity constant for combination of ferroperoxidase with CO (l') has a value of about 4×10^3 M^{-1} sec^{-1}. It is worth noting that the same value is obtained over the very large range of CO concentrations covered by the two methods employed in its determination.

The dissociation velocity constant for CO (l) calculated from L and l' according to scheme 1 is 0.001 sec^{-1} at pH 9.18. This value is not inconsistent with the upper limit of 0.01 estimated from the flash photolysis experiments. However, the rate observed in the reaction of CO–peroxidase with ferricyanide is about 0.05 and therefore much higher (50-fold) than the calculated value. The discrepancy between the rates of dissociation of CO measured with ferricyanide and those obtained using other displacing agents has been noted before for other heme proteins (Q. H. Gibson, personal communication) and suggests that in these cases the simple scheme B is oversimplified.

It is interesting to compare the affinity and rates of the reaction of peroxidase with carbon monoxide to those of other heme compounds. The affinity of peroxidase for CO is about 10-fold greater than that of free heme, and about 10-fold lower than that of myoglobin. Still larger differences are found among the same compounds in the rate of combination with carbon monoxide (Table IV). These data point out the large effect that the binding site or the environment of the heme group has on its reactivity toward the same ligand which for peroxidase is several orders of magnitude lower than that for the other heme compounds.

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