Studies on the Equilibria and Kinetics of the Reactions of Peroxidases with Ligands. III. The Dissociation of Carbon Monoxide from Carbon Monoxide Ferro-Horseradish Peroxidase*

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ABSTRACT: Carbon monoxide ferrous horseradish peroxidase dissociates very slowly in a reaction which exhibits first-order kinetics. The rate was measured by trapping the liberated ferroperoxidase with either oxygen, in which case ferric peroxidase was the product, or with nitric oxide. The rate increases with increasing pH. The heat of activation was found to be approximately 30 kcal/mole. The dissociation velocity constant (l) at 30° and pH 7.00, using NO as a trapping agent, has a value of 1.1×10^{-4} sec $^{-1}$, corresponding to a half-time of about 2 hr.

The dissociation velocity constant at 20°, estimated from this value and the heat of activation, is 1.6×10^{-5} sec $^{-1}$.

Horseradish peroxidase is a heme protein which contains one protoheme IX per molecule. In the ferric state, in which it is isolated, it forms liganded derivatives with, *e.g.*, CN^-, H_2S, F^-, N_3^-, NH_2OH, and NO, and when ferrous it forms derivatives with, *e.g.*, CO, CN^-, RNC, and NO. The spectra of these liganded derivatives as well as the spectra of the unliganded protein are very similar to those of hemoglobin or myoglobin (Keilin and Hartree, 1951). On the other hand, the reactions of peroxidase with hydrogen peroxide and with oxygen yield products different from those formed by hemoglobin or myoglobin. These differences lead us to extend our earlier study of the reaction of ferroperoxidase with the ligand carbon monoxide, which does not undergo oxidation or reduction, and in this simpler case provide a comparison between the reactions of ferroperoxidase and those of ferrohemoglobin and myoglobin. Previously we have studied the combination and equilibria of ferroperoxidase with carbon monoxide (Kertesz *et al.*, 1965) and the reaction of ferroperoxidase with oxygen (Wittenberg *et al.*, 1967).

From consideration of the rate of combination of ferroperoxidase with carbon monoxide and the affinity of ferroperoxidase for carbon monoxide it was apparent that carbon monoxide ferroperoxidase must dissociate very slowly. The dissociation velocity constant for CO (l), calculated from the equilibrium constant (L) and the combination velocity constant (l'), was 10^{-3} sec^{-1} at pH 9.18 (Kertesz *et al.*, 1965). Flash photolysis experiments indicated that this constant is lower than 10^{-2} sec^{-1}.

In the present paper the dissociation constant for CO (l) is estimated directly using either oxygen (Wittenberg *et al.*, 1967) or nitric oxide to trap the liberated ferroperoxidase. The products of these reactions are ferric peroxidase and nitric oxide ferroperoxidase, respectively. The reagents oxygen and nitric oxide are described, for convenience, as "trapping reagents," but this should not be taken to mean that the course of the reaction is necessarily understood or that the reagents are wholly without effect on the rate of dissociation of carbon monoxide.

Materials and Methods

Peroxidase. Horseradish peroxidase (HRP)[1] was a commercial product obtained from Boehringer-Mannheim (lot 06465106). Before use it was dialyzed exhaustively against water. Concentrations were determined spectrophotometrically using ϵ_{mM} (at λ 498 mμ) 10.4 for

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‡ Supported by the U. S. Public Health Service Postdoctoral Fellowship 5-F2-CA-25,058 from the National Cancer Institute.

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[1] Abbreviations used: HRP, horseradish peroxidase; CO-HRP, carbon monoxide ferrous horseradish peroxidase; NO-HRP, nitric oxide ferrous peroxidase; oxy-HRP, oxyperoxidase. L, equilibrium constant for the reaction ferro-HRP + CO $\rightleftharpoons$ CO-HRP; l', combination velocity constant for CO; l, dissociation velocity constant.

the ferric derivative. In a few experiments HRP obtained from Worthington Biochemicals Corp. (HPOFF grade) was found to dissociate CO at the same rate as the Boehringer-Mannheim product.

Carbon Monoxide Ferroperoxidase. This derivative was prepared by reduction of solutions of HRP (7×10^{-5} M) in 0.05 M potassium phosphate buffer (pH 7.0) with an exact equivalent of a solution of sodium dithionite, followed by reaction with 0.1 atm of CO gas, using the technique and precautions described previously (Wittenberg *et al.*, 1967). Solutions were protected from light.

Nitric Oxide and Carbon Monoxide. These were products of the Matheson Co.

Kinetic Experiments with Oxygen as a Trapping Agent. Solutions of CO-HRP were diluted with an appropriate buffer and were equilibrated briefly in dim light with air or oxygen at the temperature of the experiment. In this way excess CO was largely removed. It was not practical to carry out this manipulation in total darkness and some oxyperoxidase was formed by photolysis of the CO-HRP. Before beginning the measurements a period of time, often about 5–10 min, was allowed to elapse to permit decay of most of this oxyperoxidase to ferric HRP. Spectra were then recorded with a Cary Model 11 recording spectrophotometer, using 1-cm light-path cuvets held in a thermostatted cuvet holder.

Kinetic Experiments with Nitric Oxide as a Trapping Agent. Solutions of CO-HRP contained in a modified Thunberg tube fused to a 1-cm light-path cuvet were equilibrated, in the dark at 0°, with a stream of water-saturated nitrogen, to remove carbon monoxide, followed by a stream of water-saturated NO. The solutions were brought to the desired temperature in the dark and spectrophotometric measurements were begun. Rates were calculated either from the changes in extinction at λ 542 and 572 mμ or at λ 422 mμ, in which case advantage was taken of the large differences in extinction of the Soret bands of CO-HRP and NO-HRP. The spectrum in the visible region of NO-HRP has been presented (Figure 7 of Wittenberg *et al.*, 1967) and differs from that of CO-HRP. The extinction maxima of NO-HRP are 420 (ϵ_{mM} 110), 542 (10.7), and 570 mμ (10.1). The extinction maxima of CO-HRP are 422 (ϵ_{mM} 153), 542 (12.8), and 572 mμ (12.4).

Results

Kinetics of the Dissociation of CO-HRP Using Oxygen as a Trapping Agent. Figure 1 presents as an example the spectral changes occurring in the Soret region during a typical experiment, and Figure 2 presents the changes occurring in the visible region. The product is clearly ferric HRP, recognized by its absorption maxima and extinction coefficients. The spectrum of CO-HRP obtained at the end of the experiment from the identical solution by treatment with carbon monoxide and dithionite is also included in these figures. The spectral tracings obtained during the course of the reaction all pass through the isosbestic points common to ferric HRP and CO-HRP, demonstrating that no other products accumulate at detectable levels during the course of the reaction.

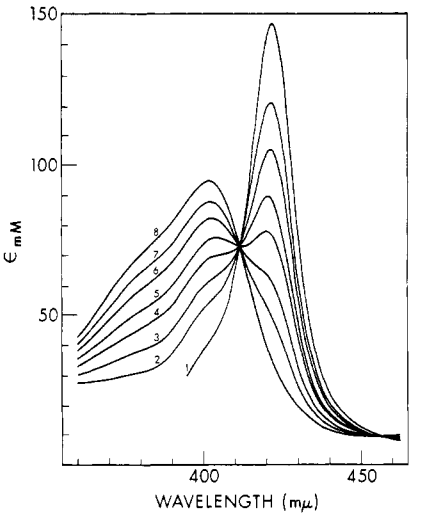

FIGURE 1. Conversion of CO-HRP to ferric HRP at 25°, in the presence of air. HRP concentration (6.3×10^{-6} M) in 0.05 M potassium phosphate buffer (pH 7.0). Trace 1 was obtained by treating the sample at the end of the experiment with dithionite in the presence of CO. Trace 2 was taken about 15 min after mixing the reagents, a delay judged sufficient to permit decay of most of the oxy-HRP formed by photolysis of part of the CO-HRP during the manipulations. Traces 3–7 were at, respectively, 30, 70, 113, 185, and 270 min after trace 2. Trace 8 was taken 25 min after exposing the sample to light to photolyze the remaining CO-HRP; oxy-HRP, formed by the photolysis, decays completely to ferric HRP during this period. Isosbestic points are at 412 and 456 mμ. The first-order rate constant is 10.7×10^{-5} sec^{-1}.

The time course of the spectral changes occurring during the conversion of CO-HRP to ferric HRP, expressed as a plot of log E_0/E *vs.* time (Figure 3), is linear, indicating a first-order dependence of the rate on CO-HRP concentration.

Figure 3 also demonstrates that the light beam of the spectrophotometer did not bring about measurable photolysis of the CO-HRP when the sample was left in the observation compartment exposed to the light beam for over 4 hr. The observed rate was not significantly changed by varying the oxygen concentration 80-fold (Table I, expt 1) and identical rates were obtained in the presence of air or oxygen.

Kinetics of the Dissociation of CO-HRP Using Nitric Oxide as a Trapping Agent. CO-HRP photolyzed in the presence of NO gives rise quantitatively to ferrous NO-HRP which is identified by its spectrum (see Figure 7 of Wittenberg *et al.*, 1967). All spectra traced during the conversion of CO-HRP to NO-HRP at pH 6.1 or 7.0 pass through the isosbestic points, λ 518 and 580 mμ, common to CO-HRP and NO-HRP, indicating that no other products accumulate at detectable levels during the

TABLE I: Apparent Rate of Dissociation of CO-HRP Measured with Oxygen as a Trapping Agent.[a]

Expt	pH	Temp (°C)	O_2 Concn (μM)	$t^1/_2$ (min)	l ($sec^{-1} \times 10^5$)
1-1	7.0	20	280	161	7.17
1-2	7.0	20	280	161	7.17
1-3	7.0	20	17	178	6.48
1-4	7.0	20	1400	161	7.17
2-1	7.0	25	280	108	10.7
2-2	7.0	25	280	108	10.7
2-3	7.0	30	1400	31	37.0
2-4	7.0	35	280	19	59.5
2-5	7.0	35	280	19	59.5
2-6	7.0	40	280	7.5	153
3-1	6.20	30	280	83	13.9
3-2	7.00	30	280	51	22.8
3-3	8.35	30	280	12	96

[a] Experiments were carried out in 0.05 M potassium phosphate buffer (pH 7.00) except for expt 3-1 and 3-3 which were carried out in 0.10 M potassium phosphate buffer. The concentration of CO-HRP was 7×10^{-6} M and the reaction was followed by the changes in extinction at 422 mμ, except for expt 2-3 in which the concentration of CO-HRP was 7×10^{-5} M and the reaction was followed by the changes in extinction at λ 542 and 572 mμ.

course of the reaction. The time course of the reaction, expressed as a plot of log E_0/E *vs.* time, is linear, indicating a first-order dependence of the rate on CO-HRP concentration. The nitric oxide concentration in all the experiments was approximately 1 atm. The observed rates (Table II) were about 2.5-fold less than the rates observed at the same temperatures and pH with oxygen as a trapping agent.

At pH 8.4 and 9.1 the spectra obtained at the end of the reaction indicated the presence of uncharacterized side products formed in addition to the main product, ferrous NO-HRP. The time course of the reaction was nonetheless linear.

The possibility was considered that dissolving NO might generate acid and affect the pH of the buffers. In separate experiments NO dissolved anaerobically in the buffers made no significant change in the pH of the buffers at pH 6.2, 7.0, and 9.14, but did change the buffer initially at pH 8.40 to a final pH of 8.3.

Effect of pH. The rate of dissociation of CO-HRP increases about six- to sevenfold from pH 6.1 to 8.4, irrespective of whether oxygen or nitric oxide is used as the agent to trap the liberated ferrous HRP. The absolute rates are different with the two trapping agents.

Effect of Temperature. The reaction rate was measured at several temperatures in order to determine the heat of activation of the reaction. Linear Arrhenius plots were obtained (Figure 4). The heat of activation is approximately 30 kcal/mole, when determined with either oxygen or NO as the trapping agent.

TABLE II: Apparent Rate of Dissociation of CO-HRP Measured with Nitric Oxide as a Trapping Agent.[a]

Expt	pH	Temp (°C)	$t_{1/2}$ (min)	l ($sec^{-1} \times 10^5$)
4-1	7.00[b]	30	121	9.56
5-5	7.00[b]	35	44.2	26.1
4-2	7.00[b]	40	17.3	66.8
5-1	6.15[c]	30	175	6.72
5-2	7.00[c]	30	94	12.3
5-3	8.40[c]	30	30.3	40.0
5-4	9.14[d]	30	28.4	40.6

[a] The concentration of NO was 1 atm in all experiments and the concentration of CO, 0.01 atm or less. The concentration of HRP was 7×10^{-5} M in expt 4 and 7×10^{-6} M in expt 5. The reaction was followed by the sum of the changes in extinction at λ 540 and 572 mμ, expt 4, or by the change in extinction at λ 422 mμ, expt 5. [b] Potassium phosphate buffer (0.05 M). [c] Potassium phosphate buffer (0.10 M). [d] Sodium tetraborate (0.05 M) (borax).

Discussion

Carbon monoxide dissociates very slowly from CO-HRP, in a reaction which exhibits simple first-order kinetics. The dissociation velocity constant for CO (l) at 30° and pH 7.00, using NO as a trapping agent, has a value of 1.1×10^{-4} sec^{-1}, corresponding to a half-time of about 2 hr. From the Arrhenius plot, presented in Figure 4, it is possible to estimate that at 20° the dissociation velocity constant would be about 1.6×10^{-5} sec^{-1}, which is 60-fold less than the value of 10^{-3} sec^{-1} previously calculated from L and l' (Kertesz *et al.*, 1965).

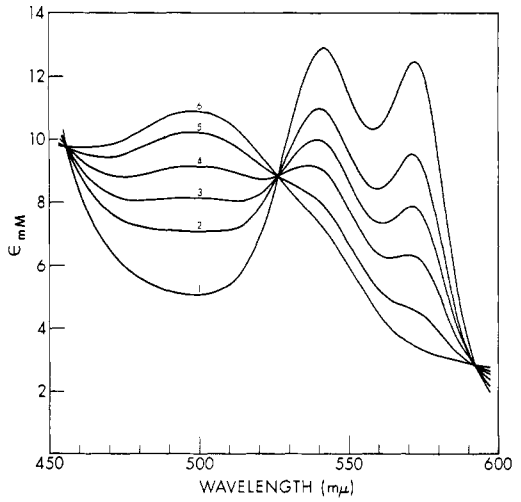

FIGURE 2: Conversion of CO-HRP to ferric HRP at 30° in the presence of oxygen. HRP concentration 7.0 × 10^{-5} M in 0.05 M potassium phosphate buffer (pH 7.0). The protocol is similar to Figure 1. Trace 1 is CO-HRP. Trace 2 is approximately 10 min after mixing reagents. Traces 3–5 were at, respectively, 15, 35, and 70 min after trace 2. Trace 6 is taken 20 min after exposing the sample to light. Isosbestic points are at 456, 526, and 592 mμ. The first-order rate constant is 37 × 10^{-5} sec^{-1}.

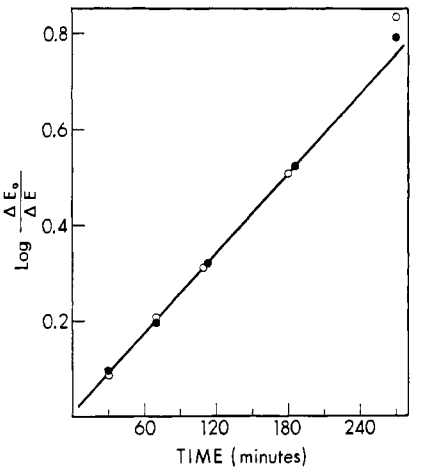

FIGURE 3: Time course of the conversion of CO-HRP to ferric HRP at 25°, in the presence of air. The reaction was followed by the sum of the changes in extinction at 400 and 420 mμ. Peroxidase concentration 6.3 × 10^{-6} M. Solid circles represent the sample exposed continuously to the light beam of the Cary spectrophotometer. Open circles represent the sample kept in dark except while actually recording the spectrum; the tracings are presented in Figure 1.

The rates of dissociation of CO-HRP obtained using NO as a trapping agent are 2.5-fold less than the apparent rates obtained with oxygen as a trapping agent. We believe that the rates measured with NO are more correct because in the presence of oxygen and at the relatively high temperatures of 20–40° ferric peroxidase is formed by more than one pathway (B. A. Wittenberg and J. B. Wittenberg, unpublished data). Nevertheless, we cannot claim to have measured with certainty the rate of dissociation of CO-HRP because of the uncertainties concerning the course of the reaction by which the "trapping agent," nitric oxide, displaces CO. The fact that the heat of activation is the same whether the rate is measured with oxygen or with NO as the trapping agent suggests that the rate-limiting step is the same in both cases.

It should be pointed out that when CO peroxidase is mixed anaerobically with ferricyanide as a trapping reagent the value obtained for the dissociation constant is about 4 × 10^{-2} sec^{-1}, at pH 7 (Kertesz *et al.*, 1965). In other words, it is about 2000 times greater than that obtained with NO. Ferricyanide is known to enter into a direct reaction with some carbon monoxide protoheme proteins, and in these cases and the present case cannot be used to measure the dissociation of carbon monoxide (Gibson and Smith, 1965; Q. H. Gibson, personal communication).

Chance (1965), using a 300-fold excess of dithionite (5 × 10^{-4} M), reports a value for l at 25° of 2 × 0^{-3} sec^{-1}, corresponding to a half-time of 6 min. Using these conditions we confirm this finding, and ascribe the rapid rate to side reactions brought about by the presence together of excess dithionite and oxygen.

Earlier we found that the equilibrium between ferrous HRP and CO was the same at pH 7.0 and 9.18, and the combination constant (l') was increased only 1.3-fold at the higher pH (Kertesz *et al.*, 1965). This implies that the dissociation velocity constant should change only slightly with pH. In fact the dissociation constant (l)

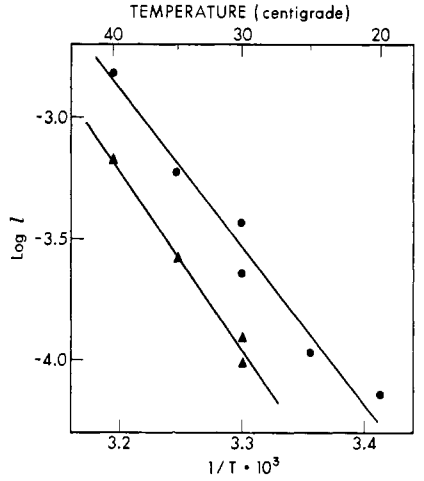

FIGURE 4: Effect of temperature on the rate of dissociation of CO-HRP. Solid circles represent oxygen as a trapping agent. Solid triangles represent NO as a trapping agent. The heats of activation found with oxygen and NO are, respectively, 30 and 33 kcal/mole.

increased three- to fourfold between pH 7.0 and 8.4–9.2. This discrepancy suggests that the assumption of a simple reversible equilibrium between ferrous HRP and CO may be over simplified.

The dissociation of carbon monoxide from hemoglobins and myoglobins is relatively rapid (half-time less than 1 min) (Gibson, 1959; Antonini, 1964, 1965; Wittenberg *et al.*, 1965) and in fact is rapid even in the case of the *Ascaris* hemoglobins which dissociate oxygen very slowly (Gibson and Smith, 1965). The extremely slow dissociation of carbon monoxide from horseradish peroxidase reported here stands in marked contrast to the behavior of the other protoheme proteins so far studied.

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Extensive Degradation of Antibody by Pepsin*

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ABSTRACT: Digestion of antibody by pepsin at pH 2.5 and 37° results eventually in degradation to small peptides and loss of antibody activity. When digestion is interrupted intermediate products of mol wt 97,000 and 47,000 can be isolated which retain antigen binding activity. The molecular weights of these products in 6 M guanidine and in the same solvent following reduction or oxidation of disulfide bonds indicate that peptide cleavages within the constituent chains have not occurred. Pepsin apparently can cleave antibody on both sides of its inter-heavy-chain disulfide bond, releasing both a dimer and monomer of the Fab region of the molecule. Further peptic digestion results in rapid degradation of these fragments to small peptides. Lower molecular weight intermediates with antibody activity could not be isolated.

γG-Globulin (γG)[1] can be cleaved by several proteolytic enzymes as well as by cyanogen bromide (Porter, 1959; Nisonoff *et al.*, 1960; Cahnmann *et al.*, 1965) in such a manner as to separate the part of the molecule associated with antigen binding activity from that related to biologic function and major antigenic identity. It appears that these enzymatic cleavages all occur at approximately the same place, about midway in the heavy polypeptide chain. Papain produces three fragments of approximately equal molecular weight. Two of these are identical, comprising the N-terminal half of the heavy chain and an intact light chain (Fab). Each carries an antibody combining site. The third fragment (Fc) is a dimer of the carboxy-terminal half of the heavy chain. The action of pepsin at pH 4.5–5 (Nisonoff *et al.*, 1960) produces a dimer of the Fab portion $(Fab')_2$ held together by a single disulfide bond and cleaves Fc in several places, producing peptides of lower molecular weight. $(Fab')_2$ has a molecular weight of approximately 100,000, contains the two antibody combining sites of the original molecule, and is capable of precipitating with polyvalent antigens. The purpose of the present investigation is to explore the further action of pepsin on $(Fab')_2$ in order to determine whether or not subfragments of lower molecular weight which are still capable of binding antigen can be isolated, and then to examine these fragments for cleavages within their polypeptide chains.

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[1] The abbreviations used are in accordance with the recommendations of the World Health Organization (*Bull. World Health Organ. 30*, 447 (1964)).