Biochimica et Biophysica Acta, 481 (1977) 279—282
© Elsevier/North-Holland Biomedical Press

BBA Report

BBA 61316

THE RATE OF REACTION BETWEEN CYTOCHROME *C* PEROXIDASE AND HYDROGEN PEROXIDE IS NOT DIFFUSION LIMITED

SHANSHA LOO and JAMES E. ERMAN

Department of Chemistry, Northern Illinois University, DeKalb, Ill. 60115 (U.S.A.)

(Received November 30th, 1976)

Summary

The apparent biomolecular rate constant for the cytochrome *c* peroxidase (EC 1.11.1.5)-hydrogen peroxide reaction has been measured as a function of temperature between 5 and 25°C at pH 4, 5.5, and 7 and as a function of viscosity over a fifteen-fold range. From the independence of the rate constant on the viscosity, it is concluded that the reaction is not diffusion limited.

We have previously determined the pH and ionic strength dependence of the rate of reaction between cytochrome *c* peroxidase (Ferrocytochrome *c*: H_2O_2 oxidoreductase, EC 1.11.1.5) and hydrogen peroxide [1]. The reaction is consistent with the mechanism shown in Eqn. 1.

$$\begin{array}{lc} & H_2CcP \\ K_1 & \uparrow\downarrow \\ & HCcP + H_2O_2 \xrightarrow{k_1} CcP \cdot I \\ K_2 & \uparrow\downarrow \\ & CcP \end{array} \qquad (1)$$

Two ionizable groups on the enzyme influence the reaction rate. The neutral hydrogen peroxide molecule reacts with the enzyme when the acidic group is unprotonated and the basic group is protonated. The apparent bimolecular rate constant is given by Eqn. 2.

$$k_1^{app} = k_1/([H^+]/K_1 + 1 + K_2/[H^+]) \qquad (2)$$

At 25°C and 0.1 M ionic strength, k_1, K_1, and K_2 are equal to $4.6 \cdot 10^7$ M^{-1} s^{-1}, $3.6 \cdot 10^{-6}$ M, and $1.5 \cdot 10^{-10}$ M, respectively [1]. The value of k_1 is comparable to values reported for other peroxidases [2—6]. The values of about 10^6 to 10^8 M^{-1} s^{-1} are somewhat below those expected for a diffusion controlled enzyme reaction, about 10^9 M^{-1} s^{-1} to 10^{10} M^{-1} s^{-1} [7]. However, steric requirements could reduce the observed rate. Hewson and Dunford [6] have

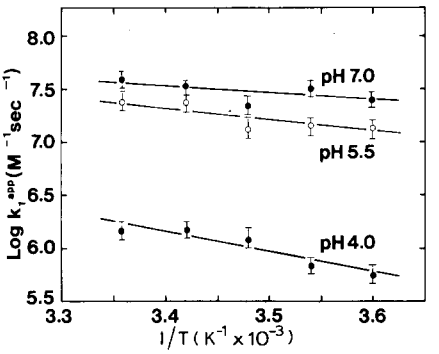

Fig. 1. A plot of the logarithm of k_1^{app} as a function of the reciprocal of the absolute temperature at three different conditions of pH. The error bars represent the standard deviation of from three to five replicate experiments at each experimental condition.

measured the temperature dependence for the horseradish peroxidase (EC 1.11.1.7)-hydrogen peroxide reaction and found that the activation energy was compatible with that for a diffusion controlled reaction. These authors suggested that the possibility of the peroxidase-hydrogen peroxide reaction being diffusion controlled should be considered.

In this report we present the results of an investigation of the temperature dependence of the cytochrome *c* peroxidase-hydrogen peroxide reaction and find that the activation parameters are very similar to those found previously for the horseradish peroxidase reaction [6]. In addition we have found that the cytochrome *c* peroxidase reaction is essentially independent of viscosity over a fifteen-fold range and conclude that the reaction is not diffusion controlled.

Isolation of cytochrome *c* peroxidase and preparation of enzyme and buffer solutions were the same as previously described [1]. In the temperature dependence studies, the buffers were 0.01 M potassium phosphate at pH 7 and 0.01 M potassium acetate at pH 4 and 5.5. The ionic strength was adjusted to 0.10 M with potassium nitrate. For the viscosity studies, sucrose-water mixtures containing 0% to 50% sucrose were prepared by weight. The mixed solvent was then used to prepare 0.01 M potassium phosphate buffers, pH 7, with potassium nitrate added to bring the ionic strength to 0.10 M. The viscosity of the sucrose-water solutions were obtained from published tables [9]. All kinetic studies were performed on a Durrum-Gibson stopped flow spectrophotometer as previously described [1].

A plot of the logarithm of the apparent rate constant as a function of

TABLE I

ACTIVATION PARAMETERS FOR THE REACTION BETWEEN CYTOCHROME *C* PEROXIDASE AND H_2O_2*

pH	E_a (kcal/mol)	$\Delta G^{\neq}$ (25°C) (kcal/mol)	$\Delta H^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (cal/deg mol)
4.0	9.3 ± 1.6	9.0 ± 0.1	8.7 ± 1.6	−0.8 ± 1.2
5.5	5.8 ± 1.1	7.4 ± 0.1	5.2 ± 1.1	−7.1 ± 0.8
7.0	2.8 ± 1.0	7.1 ± 0.1	2.2 ± 1.0	−16.3 ± 0.8

*0.100 M ionic strength.

the reciprocal of the absolute temperature is shown in Fig. 1. The activation parameters were evaluated from the plots according to standard procedures [8] and are collected in Table I. At pH 7, k_1^{app} is essentially equal to k_1*. The values of the activation parameters for k_1, 2.8 ± 1.0 kcal/mol and −16.3 ± 0.8 cal/deg mol for E_a and $\Delta S^{\neq}$ respectively, are similar to the values of 3.5 ± 1.0 kcal/mol and −12.8 ± 0.1 cal/deg mol reported by Hewson and Dunford [6] for the horseradish peroxidase reaction.

Phenomenologically, a rapid bimolecular reaction can be separated into a step where the reactants diffuse together and a step where the encounter complex reacts to form the final product as shown in Eqn. 3.

$$A + B \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} A \ldots\ldots B \xrightarrow{k_c} AB \tag{3}$$

If the encounter complex is in a steady state, the apparent bimolecular rate constant is given by Eqn. 4.

$$k^{app} = k_d k_c/(k_{-d} + k_c) \tag{4}$$

Assuming that addition of sucrose to aqueous buffers only alters the viscosity of the solution, the equilibrium constant for formation of the encounter complex, $K_d = k_d/k_{-d}$, and k_c should be independent of viscosity. (It should be noted that addition of up to 50% sucrose has no significant effect on the spectrum of cytochrome *c* peroxidase.) Both k_d and k_{-d} will depend upon the viscosity through the diffusion coefficients [7]. The value of k_d in sucrose-water mixtures is related to the diffusion rate constant in water, k_{d,H_2O}, by by Eqn. 5

$$k_d = k_{d,H_2O}\, \eta_{H_2O}/\eta \tag{5}$$

η and η_{H_2O} are the viscosities of the mixed solvent and water, respectively. Using K_d and Eqn. 5, equation 4 rearranges to Eqn. 6.

$$1/k^{app} = 1/K_d k_c + \eta/\eta_{H_2O}\, k_{d,H_2O} \tag{6}$$

A plot of the reciprocal of the apparent rate constant as a function of η/η_{H_2O} should be linear with the slope equal to the inverse of the diffusion rate constant in water. Such a plot is shown in Fig. 2.

It is apparent that k_1 is not strongly dependent upon the viscosity and we conclude that the reaction is not diffusion limited. The actual values of k_1 range from $(3.7 \pm 0.3) \cdot 10^7\ M^{-1}\ s^{-1}$ in buffer without sucrose to $(3.3 \pm 0.3) \cdot 10^7\ M^{-1}\ s^{-1}$ in 50% sucrose, a solution which has a viscosity over fifteen times higher than water. We can obtain a lower limit for k_{d,H_2O} by obtaining the maximum possible slope through the data in Fig. 2, compatible with the experimental error. The lower limit for k_{d,H_2O} is $1.8 \cdot 10^9\ M^{-1}\ s^{-1}$.

Although the rate constant measured at the lowest and highest viscosities

*The variation in activation parameters as the pH is lowered is due to the involvement of the ionization of the enzyme group at low pH in the rate determining step. At pH 4, k_1^{app} is essentially equal to $k_1 K_1/[H^+]$. The standard thermodynamic parameters for the ionization of the enzyme group with dissociation constant K_1 can be evaluated from the pH dependence of the activation parameters. These values are 6.6 ± 3.2 kcal/mol and −2.7 ± 3.2 cal/deg mol for ΔH^0 and ΔS^0 respectively. These thermodynamic values are consistent with the dissociation of a hydrogen bonded carboxylic acid or of an imidazole group.

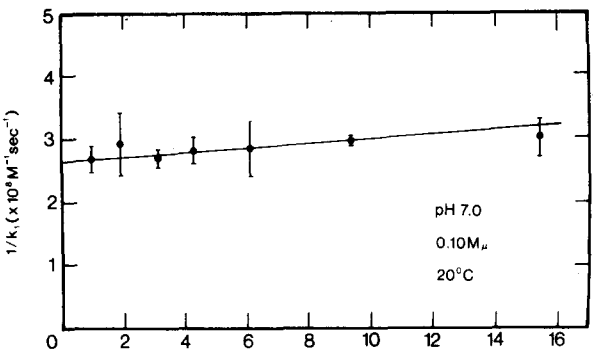

Fig. 2. A plot of the reciprocal of the apparent rate constant at pH 7 as a function of the viscosity of sucrose-water mixtures relative to the viscosity of water. The error bars represent the standard deviation for ten replicate experiments at each experimental condition.

are the same within experimental error, there is a positive slope to the data in Fig. 2. A weighted linear least squares analysis of the data gives a value of $(3.0 \pm 0.6) \cdot 10^9\ M^{-1}\ s^{-1}$ for k_{d,H_2O}. Both the "best" value and the lower limit are consistent with expected diffusion controlled rates [7] and are about two orders of magnitude larger than the observed bimolecular rate constant.

This investigation was supported (in part) by NIH Research Career Development Award GM 00249 to J.E.E. and Research Grant GM 18648 from the National Institute of General Medical Sciences.

References

1 Loo, S. and Erman, J.E. (1975) Biochemistry 14, 3467—3470
2 Maguire, R.J., Dunford, H.B. and Morrison, M. (1971) Can. J. Biochem. 49, 1165—1171
3 Marklund, S., Ohlsson, P.-I., Opara, A. and Paul, K.-G. (1974) Biochim. Biophys. Acta 350, 304—313
4 Hosoya, T. (1960) J. Biochem. (Tokyo) 47, 794—803
5 Dolman, D., Newell, G.A., Thurlow, M.D. and Dunford H.B. (1975) Can. J. Biochem. 53, 495—501
6 Hewson, W.D. and Dunford, H.B. (1975) Can. J. Chem. 53, 1928—1932
7 Eigen, M. and Hammes, G.G. (1963) in Advances in Enzymology (Nord, F.F., ed.), Vol. 25, pp. 1—38, Interscience, New York
8 Amdur, I. and Hammes, G.G. (1966) Chemical Kinetics, McGraw-Hill, New York
9 Hodgman, C.D., Weast, R.C. and Selby, S.M. (eds.) (1959) Handbook of Chemistry and Physics, 41st edn., p. 2196. Chemical Rubber Publishing Co., Cleveland