ON THE NATURE OF THE ALKALINE IONIZATION OF HORSERADISH PEROXIDASE

Navah EPSTEIN and Abel SCHEJTER
Department of Biochemistry, Tel Aviv University, Ramat Aviv, Israel

Received 19 June 1972

1. Introduction

It has been customary in hemoprotein research to attribute the alkaline ionization of high spin ferric hemoproteins to the proton dissociation of an iron bound water molecule [1]. For methemoglobin and metmyoglobin, kinetic studies of their ionization with pK 8.8 have shown that, although the overall reaction is rather complicated, it includes a step that can indeed be interpreted as the ionization of water [2–4].

Horseradish peroxidase in the ferric state undergoes an ionization with pK 10.8 that affects the redox potential of the enzyme [5] and results in a spectral change from a pure high-spin type to a type having mostly low-spin characteristics [6]. By analogy, this ionization could also be considered to reflect the unprotonation of an iron-bound water molecule. However, the kinetics of this change, described in this paper, indicate that a conformation change of the molecule appears to be involved and suggest a different mechanism as responsible for the observed ionization.

2. Materials and methods

Lyophilized horseradish peroxidase was purchased from Sigma Chemical Co. The buffers used for the different pH ranges were 0.05 M carbonate for the pH range 10.6 – 10.9; 0.05 M phosphate for the pH range 10.9 – 11.64.

pH jump experiments were performed using a Durrum stopped-flow spectrophotometer. A solution of horseradish peroxidase brought to pH 9.0 with dilute NaOH was mixed with buffer solutions at various pH's and the kinetics of the spectral change were recorded at 420 nm. All the kinetic runs resulted in first order reactions. The same pH jump experiments were carried out starting with the enzyme at pH 12.0; these gave also first order kinetics, and the rate constants for each final pH were the same, within the experimental error, whether the starting pH of the enzyme solution was 9.0 or 12.0. All the experiments were performed at 23°.

3. Results and discussion

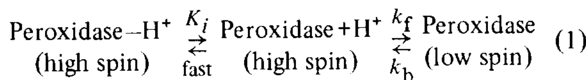
The observed first order rate constants for the spectroscopic change induced in horseradish peroxidase by changing its pH from either 9.0 or 12.0 to a final pH in the range 10.5 to 11.6, are listed in table 1. The half-times for these reactions lie in the millisecond range. Such rates are much lower than those measured for the alkaline ionization of methemoglobin and metmyoglobin [2–4]; their order of magnitude is rather similar to that of the alkaline ionization of ferric cytochrome *c*, a process that involves a conformation change of the protein [7]. Therefore, the data given in table 1 were treated in similar form to the case of ferricytochrome *c* [7].

The high spin spectrum of neutral horseradish peroxidase indicates that the sixth coordination position of its iron atom is occupied by a weak field ligand, such as H_2O or a protein carboxylate, or that it is empty. We assume that a protein side-chain unprotonates when peroxidase is brought from neutral to alkaline pH and that this is followed by a conformation change through which a protein side-chain becomes the iron ligand, thus causing the spin-type change in the spectrum. The reaction can thus be described by eq.(1):

North-Holland Publishing Company – Amsterdam

Table 1
The effect of pH on the rate of conversion of horseradish peroxidase from neutral to alkaline form.

pH	k_{obs} ($msec^{-1}$)
10.62	0.142
10.76	0.183
10.87	0.194
10.95	0.221
11.09	0.269
11.20	0.292
11.43	0.430
11.53	0.575
11.64	0.625



$$\underset{\text{(high spin)}}{\text{Peroxidase–H}^+} \underset{\text{fast}}{\overset{K_i}{\rightleftarrows}} \underset{\text{(high spin)}}{\text{Peroxidase+H}^+} \underset{k_b}{\overset{k_f}{\rightleftarrows}} \underset{\text{(low spin)}}{\text{Peroxidase}} \quad (1)$$

Such a mechanism leads to the following function:

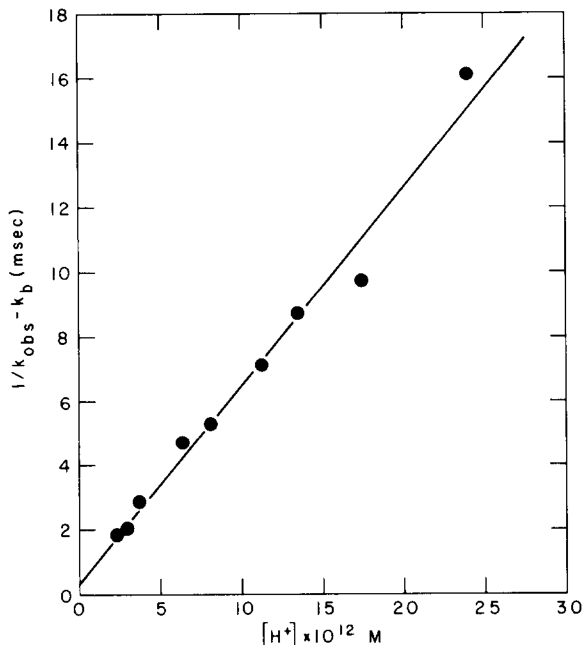


Fig. 1. A plot of the function $1/k_{obs} - k_b$ *vs.* $[H^+]$ for the alkaline ionization of horseradish peroxidase. k_{obs} and k_b are defined in the text.

Table 2
Kinetic and equilibrium constants for the alkaline ionization of horseradish peroxidase.

$$\text{Per}\cdot\text{H}^+ \overset{K_i}{\rightleftarrows} \text{Per} + \text{H}^+ \underset{k_b}{\overset{k_f}{\rightleftarrows}} \text{Per}$$

K_i	5.5×10^{-13} M
k_f	2.98 $msec^{-1}$
k_b	0.08 $msec^{-1}$
$K_c = k_f/k_b$	37.2
$K_{obs} = K_i K_c$	2.02×10^{-11} M

$$\frac{1}{k_{obs} - k_b} = \frac{1}{k_f \cdot K_i} \cdot [H^+] + \frac{1}{k_f} \quad (2)$$

where k_f and k_b are the forward and back rate constants for the conformation change described in eq.(1), k_{obs} the rate constant observed when peroxidase is brought to any final pH within the ionization region, and K_i the ionization constant of the group XH^+ [7]. K_c, the equilibrium constant for the conformation change, is given by the ratio k_f/k_b. The value of k_b, obtained by measuring directly the first order rate constant for bringing horseradish peroxidase from pH 12.0 to pH 9.0, was 8×10^{-2} $msec^{-1}$. A least-square analysis of the function $1/(k_{obs} - k_b)$ *vs.* $[H^+]$ is shown in fig.1; it is linear, with an intercept of 0.336 msec and a slope of 0.618 msec $\times$ M^{-1}.

In table 2 are listed the values of all the kinetic and equilibrium constants defined above that were estimated from the data of table 1, as represented in fig.1. It can be seen that the ionization constant of XH^+, K_i, is 5.5×10^{-13}, corresponding to a pK_i of 12.3. Furthermore, it can be shown that for values of $K_c \gg 1$, the ionization constant measured at equilibrium conditions, K_{obs}, is identical to the product $K_i K_c$. In this case, $K_i K_c = 2.0 \times 10^{-11}$, so that the observed pK for the ionization is 10.7. The pK measured at equilibrium is 10.8 [5]. The close agreement between these results provides an internal check for the correctness of the mechanism formulated.

The nature of the ionizing group, XH^+, is not know a pK of 12.3 suggests that an arginyl residue is involved, but a lysyl or a tyrosyl residue with unusually high pK's are also possibilities to consider. It is also

possible that the ionizing group, XH^+, is the same that coordinates the iron after the ionization. In any event, the results described above indicate that the heme-linked ionization of ferric horseradish peroxidase is not due to the proton dissociation of an iron-linked water molecule, and involves, most probably, the ionization of a protein side-chain with a pK = 12.3 followed by a conformation change of the protein with an equilibrium constant, K_c = 37.2.

References

[1] P.George, J.G.Beetlestone and J.S.Griffith, in: Haematin Enzymes, eds. J.E.Falk, R.Lemberg and R.K.Morton (Pergamon Press, Oxford, 1961) Part 1, p.105.

[2] A.S.Mildvan, N.M.Rumen and B.Chance, in: Probes of Structure and Function of Macromolecules and Membranes, eds.B.Chance, T.Yonetani and A.S.Mildvan (Academic Press, New York and London, 1961) Vol.II, p.205.

[3] G.Ilgenfritz and T.M.Schuster, in: Probes of Structure and Function of Macromolecules and Membranes, eds. B.Chance, T.Yonetani and A.S.Mildvan (Academic Press, New York and London, 1961) Vol.II, p.299.

[4] T.L.Fabry, J.Kim, S.H.Koenig and W.E.Schillinger, in: Probes of Structure and Function of Macromolecules and Membranes, eds. B.Chance, T.Yonetani and A.S.Mildvan (Academic Press, New York and London, 1961) Vol.II, p.311.

[5] H.A.Harbury, J.Biol.Chem.225 (1957) 1009.

[6] H.Theorell, Ark.Kemi, Mineral.Geol.16A No.3 (1942).

[7] A.Schejter, in: Probes of Structure and Function of Macromolecules and Membranes, eds. B.Chance, T.Yonetani and A.S.Mildvan (Academic Press, New York and London, 1961) Vol.II, p.451.