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UNUSUALLY FAST LIGAND EXCHANGE RATE IN HORSERADISH PEROXIDASE AS STUDIED BY TEMPERATURE-JUMP METHOD

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SUMMARY: A kinetics of azide binding by horseradish peroxidase was studied by temperature-jump method. It was found that the reaction of the enzyme with azide is quite rapid, occuring in microsecond time range. This rate is unusually rapid in contrast to the usual hemoprotein ferric iron-ligand interactions so far reported. The resulting value for the apparent association and dissociation rate constants were $k_1=6.8\times10^6$ M^{-1} s^{-1} and $k_{-1}=3.5\times10^5$ s^{-1} at 23°C and pH 5.0 for the reaction. The pH dependence of the rate constants was also studied to show a strong linkage of the ligand binding with a proton uptake of a dissociable group on the enzyme.

A number of kinetic studies on the binding of various external ligands to hemoproteins have been carried out with a relevance to their heme environmental structure and fundamental functions. The chemical exchange rate of ligation to the central heme iron so far obtained were limited to millisecond time range in ferric or ferrous iron state (1,2). We have recently found that the chemical exchange rate of azide with the ferric heme iron of horseradish peroxidase (HRP) is unusually faster than the 220 MHz proton NMR time scale, unlike the azide complexes of metmyoglobin and methemoglobin (3). This NMR study prompted us to perform a more quantitative study on the kinetics of this labile ligation of azide to the enzyme. We have found that azide binding by HRP occurs

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in microsecond time range. This ligand exchange rate is abnormally fast for the hemoproreins so far reported (1,2). The pH dependence of the apparent reaction rate constants was also studied.

MATERIALS AND METHODS: HRP was obtained from Sigma as a lyophylized sample (type VI) and was used after chromatography on a CM-cellulose column. The enzyme concentration was about 15 μM for the temperature jump measurement and was determined spectrometrically at 403 nm by using a millimolar absorptivity of 102 at pH 7.0. The solution of the enzyme and azide was made at desired pH values by employing a citric acid phosphate buffer.

The kinetic measurements were made at 23°C by a Joule heating temperature-jump apparatus, equipped with a coaxial cable as a capacitor and a 500 W Xenon lamp. A temperature jump cell of 0.5 ml capacity and 15 mm light path was used; the change in absorbance in the Soret region at suitable wave length was used to follow the kinetics. A temperature change of about 1 °C was imposed within 0.5 μs by a discharge of 60 kv.

RESULTS: Our measurements of optical absorption spectra of native HRP and its azide adduct reproduced the reported data (Soret bands at 403 and 416 nm for HRP and azide-HRP, respectively) of Keilin and Hartree (4). Temperature-jump experiment afforded fast relaxation transient traces of the spectra for the azide adduct of HRP, but not for the native HRP. Figure 1 exemplifies a typical relaxation transient observed at 388 nm at pH 5.0. This trace appears typical of a first-order exponential time dependent process, as expected for a simple bimolecular reaction (eq. 1). Figure 2 shows that the reciprocal relaxation time, τ^{-1},

$$\text{HRP} + N_3^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{HRP } N_3^- \qquad (1)$$

increases linearly with increasing the ligand concentration. The concentration of the anionic ligand was calculated from the initial concentration of NaN_3 by using a pk value of 4.7 at 23 °C for the ligand. The apparent reaction rate constants obtained from the slope and the intercept of the linear plot are $k_1=6.8\times10^6$ $M^{-1}s^{-1}$ and $k_{-1}=3.5\times10^5$ s^{-1} at pH 5.0. The resulting ratio of the rate constants ($k_1/k_{-1}=19.4$ M^{-1}) is in good accord with the literature value obtained from the optical spectra at pH 5.0 ($k_{eq}=28.6$ M^{-1}) (5).

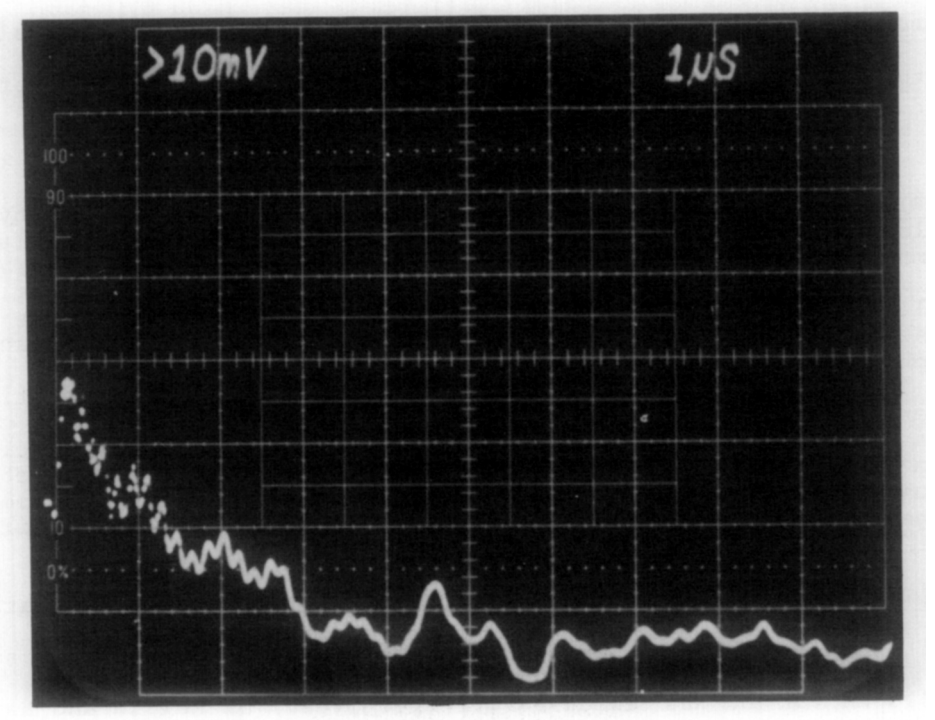

Fig. 1. A typical relaxation trace of the azide complex of horseradish peroxidase (HRP) recorded at 388 nm. The temperature of the sample solution was raised from 23 to 24 °C within 0.5 μs. The concentrations of HRP and azide are 13.6 μM and 20.0 mM, respectively in 0.05 M citric acid- 0.1 M phosphate buffer at pH 5.0. The vertical scale is transmittance increase in arbitrary unit and the horizontal scale 1 μs/div..

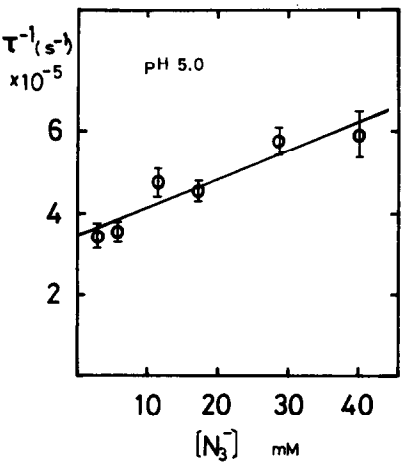

Fig. 2. Plot of reciprocal of the relaxation time vs. azide concentration. The solid line is calculated using the least square fit parameters of $k_1=6.8\times10^6$ M^{-1} s^{-1} and $k_{-1}=3.5\times10^5$ s^{-1} .

Table 1

Kinetic constants for the reaction of horseradish peroxidase and metmyoglobin with anionic ligands at 23 °C*

	Ligand	pH	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})
HORSERADISH PEROXIDASE	N_3^-	5.0	6.8×10^6	3.5×10^5
		5.5	2.9×10^6	2.0×10^5
		6.0	2.5×10^5	2.3×10^5
METMYOGLOBIN*	N_3^-	6.1	1.5×10^4	6.3×10^{-1}
	CN^-	7.0	1.7×10^2	3.0×10^{-3}
	F^-	6.1	2.0×10^1	2.9×10^{-1}
	Im**	6.2	1.0×10^2	4.7

* Data for metmyoglobin were taken from Antonini and Brunori (in "HEMOGLOBIN AND MYOGLOBIN IN THEIR REACTION WITH LIGANDS" 1971, pp. 215-285, North-Holland Publishing Company)

** Imidazole

We have also made the temperature-jump measurements at pH 5.5 and 6.0. It is revealed that the kinetic constants of the reaction are sensitive to pH variations. As table I shows, raising the pH value causes a substantial decrease in the association rate constant while the dissociation rate constant appears unchanged. In this table, the kinetic constants of metmyoglobin with anionic ligands (1) are also referred.

DISCUSSION: The azide adducts of hemoproteins such as metmyoglobin and methemoglobin have been shown to undergo temperature-dependent spin equilibria (TDSE). The dynamics of this TDSE has been measured for metmyoglobin hydroxide to show quite fast rate constant (less than 5 μs) (6,7). If this TDSE is also the case for HRP-azide (5), it seems difficult to distinguish whether the present fast time-dependent phenomena is due to ligand exchange, TDSE, or combination of both. However, the linear relation in Figure 2 shows that it is primarily caused by the fast ligand exchange, although we could not completely

rule out any contribution from TDSE. We could not observe the relaxation transient for native HRP and metmyoglobin azide under the same experimental condition examined here. This may allow us to see that TDSE if any, serves as a minor contribution to the obsrved rate constant presented here.

We have studied the azide complex of HRP in the limited pH range of 5 to 6, which is the optimum pH condition for the formation of HRP and azide complex (4,5,8). The oscilloscope trace shown in Figure 1 demonstrates an apparent relaxation time of 1 to 5 microsecond for the azide binding reaction of HRP. Although a similar fast relaxation process has been observed for the acid-alkaline transition of metmyoglobin and methemoglobin (9), the relaxation rate obtained here is salient in the usual ligand exchange reaction of hemoproteins so far reported (1,2). For example the reactions of metmyoglobin with anionic ligands occur in millisecond time range (Table 1). It is thus tempting to speculate on the structural basis for this anormalously labile azide binding by the enzyme. It is to be noted that lowering the pH value of the HRP solution leads to preferential increase in the association rate constant. This suggests a strong linkage of the ligand binding with a proton uptake of an ionizable group in the heme environment of the enzyme In the kinetics of metmyoglobin and methemoglobin, the acid-alkaline transition occuring in 1 to 10 microsecond was interpreted to associate with a protolytic reaction of the simple proton transfer from the iron-bound water molecule to the distal histidine residue followed by the ionization of the water ligand (1,9). Keeping this fast acid-alkaline transition in mind, a dissociable group at the distal side of the heme prosthetic group appears to play an important role in modulating the chemical exchange rate of the ligand with the central heme iron. It is tempting to assume that an ionizable group at the distal side of the heme is protonated in the lower pH, so that it may facilitate the fast

entry of azide to the enzyme. In other words, binding of azide by HRP could occur only when the distal ionizable group exists in the protonated form. It is, therefore, likely that the binding rate constant of azide to HRP is associated with the rate of proton uptake by this distal ionizable group on the enzyme. Although the above discussion is based on the assumption that there exists a heme linked dissociable group in HRP, many experimental evidences are now accumulated to show the presence of such a distal ionizable group (3,10,11). A question, however, bears on the different behavior of the pH dependence of the azide complexes between HRP and metmyoglobin. In the case of metmyoglobi the distal histidine residue is visualized by X-ray crystallographic study. In this case, azide binds to the ferric heme iron at any pH between 5 and 10 to produce a stable complex and the rate of reaction of azide with metmyoglobin is much slower than that for HRP. One possibl explanation for this different reactivity of HRP and metmyoglobin with azide could be drawn from the difference of the spacial location or orientation of the distal ionizable group in these two hemoproteins.

The unusually fast ligation of azide is quite characteristic of ferric HRP, in contrast with other hemoproteins such as metmyoglobin, methemoglobin and cytochrome c peroxidase. Further studies with isoenzyme of HRP and Japaneseradish peroxidase are now in progress.

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