CYANIDE AS A POSSIBLE LIGAND OF LOW-SPIN PEROXIDASES IN PLANT TISSUES.

I. Yamazaki, R. Nakajima, H. Honma and M. Tamura
Biophysics division, Research Institute of Applied Electricity
Hokkaido University, Sapporo, Japan

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Horseradish paraperoxidase was first discovered by Theorell in 1940 and later found to be a derivative of normal peroxidase by Theorell (1947), Keilin and Hartree (1951) and Paul (1958). Paraperoxidase is a red, low-spin hemoprotein and gives hemochromogen type absorption bands on reduction with $Na_2S_2O_4$. We found (1966) that by addition of $HgCl_2$ or PCMB, paraperoxidase turned to a brown, high-spin peroxidase similar to normal peroxidase. It was, once, assumed that a certain component, possibly a sulfur compound was responsible for the conversion between low-spin and high-spin peroxidases. The characteristic features of the component are as follows. 1. It combines stoichiometrically with paraperoxidase in which the component has been removed (component-free paraperoxidase) to give back paraperoxidase. 2. The component designated herein as (F) combines with mercaptide-forming reagents to form $Hg^{++}(F)_2$, PCMB(F) and $Ag^{+}(F)$. 3. The component evaporates from either neutral or acidic solution of the mercuric salt on addition of slightly excess cysteine. 4. There is no characteristic absorption for this component in the ultraviolet and visible range. 5. It gives a negative nitroprusside test and has no catalytic effect on the iodine azide reaction as mercaptans do. The component is finally identified as cyanide from the results of a positive cupric-benzidine test in the gas phase. It was concluded that this component-

free paraperoxidase has an extraordinarily high affinity for cyanide in the ferrous form as well as in the ferric form.

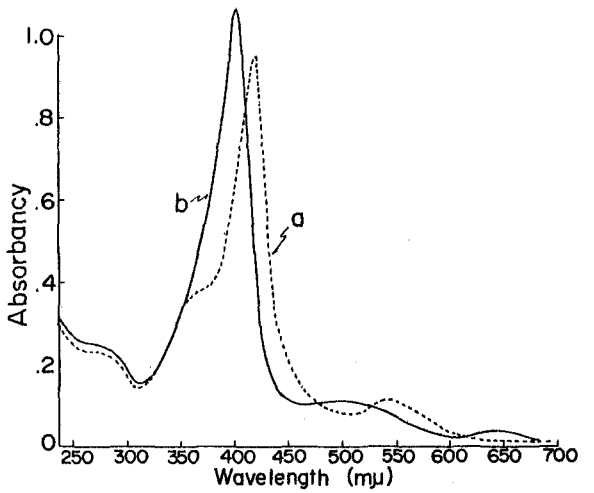

Fig. 1. Reversible conversion of horseradish paraperoxidase into normal type peroxidase by $HgCl_2$. 0.05 M K- and Na-phosphate buffer (pH 7.0). The enzyme was crystallized three times from ammonium sulfate solution. a; paraperoxidase. b; a + 8 μM $HgCl_2$. Absorption spectrum a can be regenerated upon addition of approx. 20 μM cysteine to the solution of b, except for the ultraviolet region.

Horseradish paraperoxidase was very easily crystallized from ammonium sulfate solution in the early stage of purification and the R.Z. value (E_{418}/E_{280}) reached 4.1 after three crystallizations (Fig. 1). Component-free paraperoxidase could be easily prepared from paraperoxidase by ammonium sulfate fractionation in the presence of $HgCl_2$ and crystallized in the same manner as that for normal paraperoxidase. Component-free paraperoxidase has the same absorption spectrum (high-spin type) as that of normal peroxidase both in the ferric and ferrous form, but reverts to the original spectrum of normal paraperoxidase (low-spin type) upon addition of a stoichiometric amount of the component previously removed from the enzyme or free cyanide ion as shown in Fig. 2. Paraperoxidase is stable at pH 4.5 in the ferric state but changes into a high-spin peroxidase at slightly acidic pH's in the ferrous state (Fig. 3). The reaction of cyanide with the bivalent iron of heme, hemoglobin

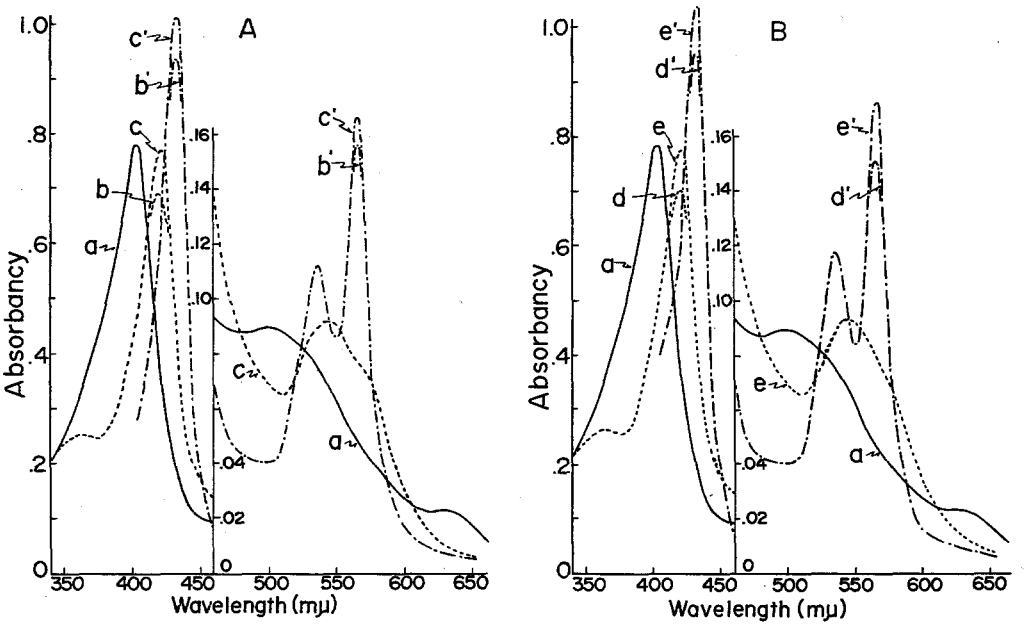

Fig. 2. Regeneration of paraperoxidase from component-free paraperoxidase by the addition of component (A) and KCN (B). 0.05 M K- and Na-phosphate (pH 7.0). Component-free paraperoxidase was prepared from the paraperoxidase by ammonium sulfate fractionation in the presence of $HgCl_2$ and crystallized from ammonium sulfate solution. The component was driven out of the above supernatant by N_2 bubbling with the addition of excess cysteine and was fixed as $Hg(F)_2$ by passing the gas into a $HgCl_2$ solution. a; Factor-free paraperoxidase. b; a + 3.5 μM $Hg(F)_2$ with 20 μM cysteine. c; a + 7 μM $Hg(F)_2$ with 20 μM cysteine. b'; b + excess $Na_2S_2O_4$. c'; c + excess $Na_2S_2O_4$. d; a + 8 μM KCN. e; a + 11 μM KCN. d'; d + excess $Na_2S_2O_4$. e'; e + excess $Na_2S_2O_4$.

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and myoglibin has been known (Drabkin, 1942, Kaziro et al, 1956 and Keilin, 1955). Keilin and Hartree (1955) found that cyanide reacted with ferroperoxidase, forming a much more stable compound than with hemoglobin and myoglobin. According to them, the dissociation constant of ferroperoxidase-cyanide is about 1.3 mM and is about 1000 times larger than that of ferriperoxidase-cyanide. They also observed that on acidifying a solution of ferroperoxidase-cyanide the hemochromogen type bands gradually disappeared. The pH dependence of the stability of paraperoxidase in the ferrous state had the same pattern as those of cyanide complexes of myoglobin and normal ferroperoxidase although the dissociation constants for these cyanide complexes are greatly different; M order for myoglobin, mM order for normal ferroperoxidase and

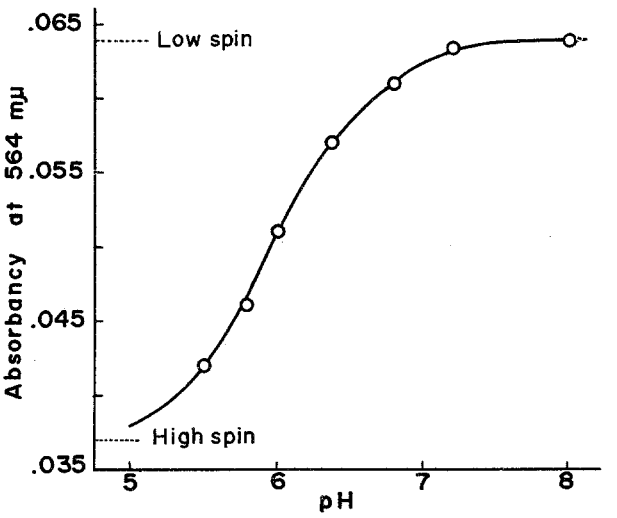

Fig. 3. The pH dependence of the stability of reduced horseradish paraperoxidase. The intensity of α-band of reduced paraperoxidase was plotted against pH. 0.05 M K- and Na-phosphate.

μM order for component-free ferroparaperoxidase at neutral pH.

Low-spin peroxidases like horseradish paraperoxidase have been isolated from sweet potato (Kondo and Morita, 1952), broad beans (Morita, 1954), Japanese radish (Yamazaki and Fujinaga, 1956 and Morita and Kameda, 1957) and wheat germ (Hagihara et al, 1958). Our interest was to confirm whether or not the same ligand is involved in these low-spin peroxidases. A low-spin peroxidase "Peroxidase 566" was prepared from wheat germ according to the modified method of Hagihara et al (1958). Extract of wheat germ with slightly alkaline water was treated with Amberlite CG-50. Adsorbed peroxidase 566 was eluted with 0.5 M ammonium phosphate (pH 7.5) and purified by ammonium sulfate fractionation and CG-50 chromatography. The absorption spectrum of peroxidase 566 is shown in Fig. 4. This enzyme clearly showed the conversion from low-spin to high-spin upon addition of a stoichiometric amount of $HgCl_2$ and the conversion mechanism was found to be the same as that of horseradish paraperoxidase. The conversion component of wheat germ peroxidase 566 was then identified as cyanide again as a result of it giving a cupric-benzidine test.

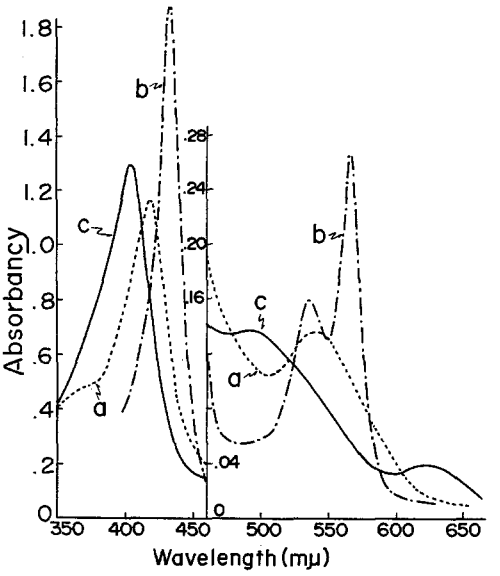

Fig. 4. Absorption spectra of wheat germ "peroxidase 566". 0.05 M K- and Na-phosphate (pH 7.0). a; peroxidase 566. b; a + $Na_2S_2O_4$. c; a + 20 μM $HgCl_2$.

It is worthwhile to point out here an early finding by Hagihara et al (1958) that their peroxidase 566 showed clear α- and β-bands only in the presence of cyanide when reduced with $Na_2S_2O_4$. They suggested that this might be due to the protection by cyanide from autoxidation. Later, Shin and Nakamura (1951) prepared peroxidase 566 in the complete form which gave clear α- and β- bands on reduction with $Na_2S_2O_4$ in the absence of added cyanide. These results are now easily explained by the fact that the ligand of peroxidase 566 is cyanide and is separable during unfavorable preparation procedures such as acidification and prolonged dialysis of the enzyme solution.

The ligand of most of the low-spin peroxidases prepared from plant tissues is probably cyanide since we found that this component of horseradish paraperoxidase was common to the low-spin peroxidases from Japanese radish and mung bean seedlings as well. It now remains to answer the question as to whether cyanide coordinates with the peroxidases in the intact plant tissues or comes from some external source during the preparation procedure. In the case of wheat germ the later possibility can probably be neglected by the fact

that Tagawa and Shin (1959) observed a strong broad band at 560-570 mμ from reduced wheat germ paste and our very simple preparation procedure (adsorption of the enzyme with CG-50 from wheat germ extract) could show the existence of peroxidase 566. Plant peroxidases may fall into two major groups, high-spin peroxidases (metmyoglobin type) and low-spin peroxidases (cytochrome b type). Horseradish paraperoxidase and wheat germ peroxidase 566 belong to the later group and may be called cyanoperoxidase. Further experiments are necessary to confirm the existence and the physiological role of the cyanoperoxidase in plant tissues.

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