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A soluble hemoprotein 563 isolated from spinach leaves

Horseradish paraperoxidase[1] and wheat germ peroxidase 566 (ref. 2) are low-spin peroxidases giving a hemochromogen type of absorption spectrum on reduction with $Na_2S_2O_4$. The α-bands lie at 564 mμ in the horseradish paraperoxidase and at 566 mμ in the wheat germ peroxidase 566. We have found that the ligand of both peroxidases is cyanide and have suggested that this ligand is common to all low-spin peroxidases isolated from plants such as sweet potato, broad beans and Japanese radish[3,4]. The absorption spectra of these peroxidases are very similar to those of the *b* group of cytochromes, and there is some possibility of confusion between these two groups of hemoproteins. In spinach leaves, for instance, there are two hemoproteins having α-bands at 563 mμ, and they cannot be distinguished from each other in respect of their redox difference spectra. One of them is present in an "insoluble" form in chloroplast which was found in 1954 by HILL[5], who named this component cytochrome b_6. The other is a soluble hemoprotein but has not yet been identified. We have prepared this soluble hemoprotein as follows.

300 g spinach leaves were homogenized with 900 ml of 0.5 M sucrose solution in 0.1 M phosphate buffer (pH 7.4). The juice was squeezed through gauze and centrifuged for 10 min at 1500 $\times$ *g*. The precipitate (chloroplast) was decolorized with 80% acetone (—20°) and suspended in 0.1 M phosphate solution (pH 7.4). This preparation was used for spectrophotometric measurements of cytochrome b_6. The soluble hemoprotein was precipitated from the chloroplast-free supernatant by ammonium sulfate fractionation (from 0.2 to 0.9 saturation). The precipitate was dissolved in a small volume of water and dialyzed against 0.005 M phosphate buffer (pH 7.4). The hemoprotein was then adsorbed on to an Amberlite CG-50 column which had been equilibrated with the same buffer. The fraction eluted with 0.05 M phosphate buffer (pH 7.4) was further fractionated with ammonium sulfate (from 0.4 to 0.7 saturation).

The absorption spectra of this purified hemoprotein are shown in Fig. 1. The hemoprotein showed a clear α-band at 563 mμ but only a shoulder around 530 mμ when reduced with $Na_2S_2O_4$. The typical hemochromogen bands could be observed only in the presence of added cyanide. This is a similar phenomenon to the one reported by HAGIHARA *et al.* in wheat germ peroxidase 566 (ref. 2) and common to the low-spin peroxidases isolated from plant tissues[4]. Like low-spin peroxidases this hemoprotein was also converted into a high-spin state by the addition of a stoichiometric amount of mercuric salt. The soluble hemoprotein 563 in spinach leaves had about a half of the peroxidase activity of horseradish peroxidase and may be termed "cyanoperoxidase" as well as horseradish paraperoxidase and wheat germ peroxidase 566. The dissociation constants of the spinach peroxidase–cyanide complex were 0.32 μM and 3 μM in the ferric and ferrous states, respectively.

Our interest was then to confirm whether or not there was any relationship between cytochrome b_6 in chloroplast and spinach "cyanoperoxidase". However, unlike the soluble hemoprotein no significant changes could be observed in the absorption spectrum of cytochrome b_6 when acetone-treated chloroplasts were subjected to the following treatments: (1) addition of 0.1 mM $HgCl_2$ or *p*-chloromercuribenzoate solution before and after treatment with 0.2% deoxycholate; (2) sonication in the

presence of 0.1 mM $HgCl_2$; and (3) washing several times with diluted $HgCl_2$ solution. Although the accessibility of these chelators for the cytochrome in chloroplast is unknown, it may be concluded that the ligand of cytochrome b_6 is not cyanide and that the soluble hemoprotein with an α-band at 563 mμ is quite different from cytochrome b_6 in chloroplasts.

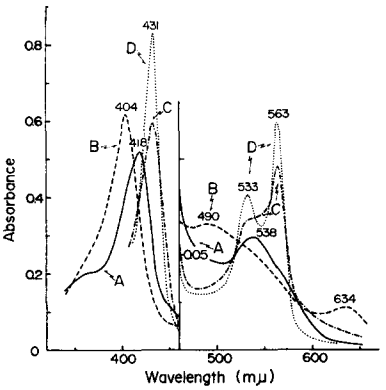

Fig. 1. Absorption spectra of spinach leaf peroxidase in 0.05 M phosphate (pH 7.0). A, purified peroxidase (about 80% of the enzyme was complexed with cyanide); B, cyanide-free peroxidase (A + 25 μM $HgCl_2$); C, reduced form of enzyme, A (A + $Na_2S_2O_4$, about 50% ferroperoxidase was complexed with cyanide); D, ferroperoxidase–cyanide complex (C + 50 μM KCN).

"Cyanoperoxidases" have been isolated from a variety of plant tissues. ABROL[6] has suggested the occurrence of free hydrocyanic acid in some plants, and it has been reported that young seedlings of a number of plant species incorporate hydrocyanic acid into asparagine or γ-glutamyl β-cyanoalanine to a considerable extent[7,8]. We have recently isolated "cyanoperoxidase" from mung bean seedlings. It has yet to be determined, however, whether or not cyanide combines with these hemoproteins in intact plant tissues.

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